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Serum Lipids and Urinary Estrogens of Non-Pregnant Menstruating Young Women

Shiao-fan Lee
Utah State University

SERUM LIPIDS AND URINARY ESTROGENS OF NONPREGNANT MENSTRUATING YOUNG WOMEN

by

Shiao-fan Lee

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Shiao-fan Lee

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ABSTRACT

Serum Lipids and Urinary Estrogens of Nonpregnant Menstruating Young Women

by

Shiao-fan Lee, Doctor of Philosophy

Utah State University, 1971

Major Professor: Dr. E. B. Wilcox
Department: Nutrition and Biochemistry

Twelve university women students served as experimental subjects in a study of the serum lipids and urinary estrogens of healthy nonpregnant menstruating women, who were living under their usual conditions.

The subjects maintained constant weight on their ordinary diets during the entire study period. Antecubital blood and 24-hour urine specimens were collected on certain days which represented different stages of the menstrual cycle. Quantitative analyses were made on serum total cholesterol, lipid phosphorus (phospholipids), triglycerides and total lipids. Gas-liquid chromatographic analysis of the fatty acid composition of each serum lipid component was also made. Urinary estrone, 17β-estradiol and estriol were separated and quantitatively determined by chromatographic and spectrophotometric techniques.

Basic data on serum lipid levels, composition of the fatty acids of cholesterol esters, phospholipids and triglycerides and urinary estrogens were obtained on these young women. Findings included the following:

1. Mean values of serum total cholesterol, phospholipids, triglycerides and total lipids were 162, 165, 105 and 544 mg per cent, respectively.

The interindividual variation was greater than intraindividual variation. The values of triglycerides were more variable than those of cholesterol and phospholipids.

2. The major fatty acids in lipid fractions were palmitic, stearic, oleic and linoleic. The highest amounts of fatty acid in cholesterol esters, phospholipids and triglycerides were linoleic, 51; palmitic, 28; and oleic, 33 per cent, respectively. Inter- and intra-individual variations were high.

3. The urinary estrogen values showed that 17 β-estradiol (E_2), was usually present in the least and esteriol (E_3), in the greatest amounts. The mean values of E_1 (estrone), E_2, E_3 and E_t (total) were as follows: 8.7, 4.8, 16.4, and 29.9 μg per 24-hour urine.

4. The menstrual cycle did affect the urinary excretion of estrogens which showed the lowest values during the first week and then rose to a peak which occurred on or about the time of ovulation or mid-cycle. Then it fell and rose again between the third and fourth week of the cycle. The second peak was usually lower than the first one.

5. Cyclical changes of the concentrations of serum total cholesterol, phospholipids and total lipids have been observed. These changes appeared to be influenced by the estrogenic hormonal activity of the menstrual cycle. The increased excretion of urinary estrogens with a decreased (negative correlation) concentration of serum lipids was recognized.

6. Linoleic acid in cholesterol esters, as well as palmitic acid in phospholipids were found in cyclic changes. The patterns were quite similar to those of serum lipids.

(180 pages)

INTRODUCTION

Experimental data bearing on the influence of hormones on carbohydrate and protein metabolism are well documented (See Friedman, 1968; Scanu, 1965). In contrast, information concerning hormonal influences on lipid metabolism is limited. Although only fragmentary data on the mechanisms involved in the hormonal control of lipid metabolism are currently available, there is ample evidence that aging, the menopause, pregnancy, diabetes, hypothyroidism, ovariectomy and androgens raise blood cholesterol, while thyrotoxicosis, ACTH, cortisone and estrogens lower it (Furman et al., 1958; Hess, 1964; Stamler et al., 1959, 1960 and Turner, 1963). Under these circumstances attention has been focused on the fact that the endocrine system might play an important regulatory or mediating role in these mechanisms.

Evidence now shows that the gonads exert an important influence on the level of circulating lipids. The comparatively rare occurrence of coronary artery disease in women during the reproductive phase is well known. Bilaterally ovariectomized women show a higher incidence of coronary artery disease than normal women of corresponding ages. Sex differences in the distribution of serum lipids have long been reported. It has been demonstrated that the male has the greatest increase in the early thirties, whereas the female maintains her youthful lipid level longer and does not reach her peak serum lipid level until the age of fifty or later. This difference must, at least in part, be influenced by female sex hormones (See Friedman, 1968 and Masoro, 1968).

The control of hypercholesterolemia in men and in women after the menopause by estrogen administration has been recognized (Hess, 1964; Moses, 1963; Musa et al., 1965; Stamler et al., 1959, 1960).

One cardinal question for therapy in hyperlipemia (atherosclerotic disease) is the utility of hormones as a therapeutic agent. Equally important is the problem of the role of hormone in the etiology of human disease. Attempts at rational therapy thus far have, perhaps, ignored the pro-atherosclerotic effect of certain endocrine states.

In the past two decades the conquest of infectious diseases and improvements in diagnosis and treatment of other diseases have resulted in the establishment of atherosclerotic heart disease as the major cause of death in the western world. Indeed, in the United States more than 50 per cent of all deaths are attributed to cardiovascular causes. Hence, the attention of many workers has been directed towards the investigating of lipid metabolism in coronary artery disease. An elevation of the plasma lipids has frequently been reported in patients with coronary artery disease when compared with normal (Stamler et al, 1959, 1960, 1963; Scanu, 1965). It has been postulated that if the plasma lipids were involved in the pathogenesis of coronary artery disease, and since women are relatively immune before the menopause, further information concerning the relationship between estrogens and plasma lipids might be obtained by studies during the normal menstrual cycle.

Preliminary studies in apparently healthy menstruating females suggest that estrogen excretion increases twice during the menstrual cycle: the

first coincides with ovulation, while the second occurs during the luteal phase of the cycle.

In the few studies that have been made of the relation of blood lipids to the menstrual cycle, the response of the lipid levels has resulted in contradictory findings by several investigators (See Oliver and Boyd, 1953, Hess, 1964). Further detailed investigations of plasma lipids and urinary estrogen levels during the normal menstrual cycle are essential.

The present study was designed to obtain basic information on the levels of the excretion of urinary estrogens, serum lipids and the fatty acid compositions of each lipid fraction during the menstrual cycle. These findings on normal healthy young women, under normal home conditions could form a base for interpretation to delineate their role(s) in abnormal metabolism of lipids found in patients with atherosclerosis and coronary heart disease.

REVIEW OF LITERATURE

Serum Lipid Levels

Although most of the lipid in serum is present in lipoprotein complexes, over the years much of the attention of investigators has been focused on serum lipids rather than lipoproteins (Fredrickson, Levy and Lees, 1967). It is therefore necessary to have a knowledge of serum lipid levels, and whenever possible, to relate this information to lipoprotein structures (Fisher and Gurin, 1964).

Lipids in serum occur mainly as cholesterol esters, triglycerides (or neutral fat), phospholipids, free fatty acid and free cholesterol (Friedman, 1968). In the literature, the term lipemia or hyperlipemia is often used; refer to an elevation of blood lipids above normal levels (Masoro, 1968). Blood lipid levels are affected by diet but under most conditions are extremely uniform in any one individual. Moreover, the composition of fasting blood of different healthy or normal individuals shows quite constant values (Friedman, 1968). However, difficulty in defining normal serum lipid values results from the influence of various factors on serum lipid concentrations. Cholesterol and phospholipid concentrations vary with age and show an increase from the third to the sixth decade followed by a slight decrease thereafter (Adlersberg et al., 1956; Lewis et al., 1957; Moses, 1963). In higher decades, above 70 years of age, values may be influenced by the inclusion of persons with clinically silent disease (Wagener, 1967). Furthermore, alimentary influences result from total

amount of ingested fat and the composition of dietary fatty acids (Friedman, 1968). Moreover, it is varied with sex differences as well as with altered physiological conditions. The literature contains numerous reports on investigations of serum cholesterol which have been extensively reviewed (Cook, 1958; Feldman, Benkel and Nayak, 1963; Nestel, 1970; Rothblat, 1969; Waxler and Craig, 1964). Fewer data are available on the other lipid classes, but the importance of simultaneous investigations of all lipid components has been recognized, particullarly in recent years (Albrink and Man, 1959; Cromie et al., 1963; Fredrickson et al., 1967; Herman, Zakim and Stifel, 1970; Waxler and Craig, 1964).

Values for total lipid and the distributon of lipid classes, which have been reported by some investigators are presented in Table 1. Most mean values (expressed in mg per cent) for total lipid recorded over a thirty year period ranged from 385 to 675. Higher mean concentrations of 735 (Page et al., 1935), and 836 (Kornerup, 1950), however , have been reported. Albritton (1952), cited 397 to 722 as the range of standard values for humans. Slightly more than 30 per cent of the total lipids usually are cholesterol esters; approximately 30 per cent, phospholipids; 20 per cent triglycerides; 10 per cent, free cholesterol; and two to three per cent of the total lipid, free fatty acids (Hallgren et al., 1960; Masoro, 1968).

The fatty acid content of cholesterol esters, phospholipids and triglycerides of serum obtained by several investigators are summarized in Table 2. Most of the values reported for the major fatty acids, palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), linolenic (18:3) and arachidonic (20:4), showed good agreement. However, values which differ markedly often have

Table 1. Total lipids and the distribution of lipid in serum as reported in the literature

Sex	Total lipid mg/100 ml	Lipid classes: Cholesterol esters	Free cholesterol	Triglycerides plus free fatty acids	Phospholipids	Reference	Year
		Percent total lipid					
M							
W	659				34	Man & Peters	1933
M							
W	595	35	9	25	31	Boyd	1935
M	735	34	11	31	25	Page et al.	1935
		38.3	14.1	23.3	22.8	Weinhouse & Hirsch	1940
M							
W	836	32	7		21	Kornerup	1950
M							
W	591	37	10	18	36	Wilmot & Swank	1952
M							
W	530	34	9	27	31	West & Todd	1957
M							
W		39	8	16	32	Luddy et al.	1958
M							
W	588	44	12	16	34	Hallgren et al.	1960
M							
W	635	47	4			Lund et al.	1961a
M							
W	568	62	5				1961b
M							
W	629	34	10	14	35	Svanborg & Svennerholn	1961
W	587			14		Waxler & Craig	1964
M							
W	603	37	8	24	31	Smith	1965
W	602	37	8	22	34	Sang	1967

Table 2. Major fatty acid values of lipid classes in serum as reported in the literature

Lipid class	Per cent Fatty Acids					Reference
	16:0	18:0	18:1	18:2	20:4	
Cholesterol esters	11-18	tr-4	19-30	40-52	4-8	Hallgren et al., 1960; Hanahan et al., 1960; Luddy et al., 1958; Michaels et al., 1958; Schrade, 1961; Scott et al., 1964; Smith, 1965; Swell et al., 1962; Tuna and Mangold, 1963; Tuna et al., 1958;
Triglycerides	25-31	4-7	36-45	9-14	tr-2	Hallgren et al., 1960; Luddy et al., 1958; Schrade, 1961; Smith, 1965; Swell et al., 1962; Tuna and Mangold, 1963.
Phospholipids	28-37	12-16	12-19	14-24	4-10	Dole et al., 1959; Hallgren et al., 1960; Schrade, 1961; Scott et al., 1964; Smith, 1965; Swell et al., 1962.

been reported. Hanahan, Watts and Pappajohn (1960) found ten per cent arachidonic acidin serum triglycerides whereas other investigators found only two per cent or less in this fraction. Oleic acid in phospholipids was reported by Luddy et al. (1958) as 36 per cent, but these values were twice as much as values found generally by other workers. The proportions of fatty acids reported appear to be characteristic of each class.

The data in Table 2 may be summarized as follows: in cholesterol esters, approximately half of the fatty acids were linolenic; one-fifth, oleic; and a little more than one-tenth, palmitic; in phospholipids, palmitic acid predominates with values equaling one-third, while one-half of the total fatty acids were present as stearic, oleic and linoleic acids in relatively comparable amounts. In triglycerides, most of the fatty acids were present as palmitic and oleic, with slightly higher concentrations of oleic than palmitic and only about one-tenth was linoleic acid.

Urinary Estrogen Levels

The sources of estrogens in the organism are the ovaries, the testes, the placenta and the adrenals as well (Dale, 1967, and Turner, 1963). Some estrogens are also of plant (date seed) origin (Heftmann, Ko and Bennett, 1965).

The predominating natural estrogens of the human are 17β-estradiol, estrone and estriol. Many other estrogen metabolites with estrogenic activity have been isolated and identified in significant amounts from human urine. Those have recently been reviewed by Lee (1967) and are listed in Table 3.

Table 3. Estrogen isolated from human urine[a]

Substance	Reference	Year
Estrone	Doisy et al.	1929
	Buntenandt	1929
Estriol	Marrian	1930
	Doisy et al.	1930
	Doisy and Thayer	1931
17β-Estradiol	Smith et al.	1939
	Huffman et al.	1940
16-Oxo-estrone	Serchi	1953
16-epi-Estriol	Marrian and Bauld	1955
6-Hydroxy-estrone	Loke et al.	1957
16α-Hydroxy-estrone	Marrian et al.	1957
2-Methoxy-estriol	Fishman et al.	1958
2-Methoxy-estrone	Loke and Marrian	1958
16β-Hydroxy-estrone	Layne and Marrian	1958
16-Oxo-17β-estradiol	Layne and Marrian	1958
18-Hydroxy-estrone	Loke et al.	1958
		1959
16, 17-epi-Estriol	Breuer and Pangels	1959
2-Methoxy-17β-estradiol	Frandsen	1959
17-epi-Estriol	Breuer	1960
2-Hydroxy-estrone	Notchev and Stimmel	1962

[a]See Lee, 1967.

In females, estrogens appear in detectable amounts in urine at the age of 8 to 11, increase with menarche, fluctuate predictably with the menstrual cycle, rise greatly during pregnancy and fall after menopause. In certain pathological states the estrogen values vary drastically (Nathanson, Towne and Aub, 1941 and Turner, 1963).

Other than in pregnancy and in pathological states in which very high levels prevail, the techniques for analysis of estrogen fractions in blood are yet insufficiently developed to have provided precise information on the physiological levels and fluctuations in blood (Wotiz and Clark, 1970).

Information on the levels of the three classic estrogens obtained in urine indicates that in healthy females 17β-estradiol is usually present in least amounts and that estrone and estriol excretion rates are about twice that of 17β-estradiol. Values for total estrogens and the individual estrogens which have been reported for other workers are presented in Table 4.

Studies of apparently healthy menstruating females suggest that excretion of the three estrogens mentioned above is least during the first week of the cycle and then rises to reach a peak at about the time of ovulation, probably decreases transiently and then rises to a second peak between the third and fourth week of the cycle. With the onset of menses the low levels again appear (Brown, 1960; Lee, 1965, 1967; Loraine and Bell, 1963; Turner, 1963).

The relative amounts of the three estrogens excreted in the urine during the follicular and luteal phases of the menstrual cycle are expressed as ratios of 17β-estradiol to estriol and are 15:40:45 and 14:38:48, respectively (Brown, 1960).

Table 4. Urinary estrogen values of normal humans as reported in the literature

No. of Subjects	Age	Estrone	17β-estradiol	Estriol	Total estrogen	Reference	Mark
		Range (Mean) μg/24-hour urine					
Female							
	Healthy girls' low basal levels increase at age 8-11 years and cyclic fluctuations appear					Nathonanson, Towne & Aub 1941	
16	18-41	4-7 (5)	0-3 (2)	0-15 (6)	4-30 (14)	Brown	Menses, week 1
16	18-41	11-31 (20)	4-14 (9)	15-34 (27)	35-100 (57)	1955	Menses, Ovulation
16	18-41	10-23 (14)	4-10 (7)	8-72 (22)	5-82 (33)		Menses, week 3-4
		(7.8)	(3.8)	(27.0)		Bauld, 1956	Day 9 of cycle
10	18-37	1.2-11.0 (4.5)	0-7.7 (1.7)	2.1-12.4 (5.4)		Loraine	menstruation
10	18-37	1.4-18.0 (5.7)	0-13.0 (2.9)	1.5-17.6 (6.8)		and	follicular phase
10	18-37	2.5-24.6 (12.6)	1.0-23 (5.2)	2.7-73.8 (17.8)		Bell	ovulatory phase
10	18-37	2.2-23.6 (9.4)	0-13.2 (3.6)	4.0-58.7 (15.0)		1963	luteal phase
5	20-21	0.8-7.3 (4.5)	0-0.5 (2.8)	4.8-27.8 (9.6)	9.4-38.7 (20.9)	Lee	Menses, week 1
5	20-21	3.7-13.7 (9.2)	1.1-12.7 (3.8)	7.4-63.8 (24.4)	12.9-80.4 (37.3)	1967	Menses, Ovulation
5	20-21	1.9-11.2 (6.6)	1.4-9.4 (2.7)	3.2-26.8 (22.2)	14.6-39.4 (28.7)		Menses, week 3-4
5	20-21	(6.9)	(18.5)	(18.5)	(29.9)		Mean values of the entire cycle
Male							
					4-60	Segre and Lobotsky 1964	
		3-21				Engel, 1950	
30	25-55	1-11 (6)	0-7 (2)		9-25 (14)	Cameron, 1957	
					4-25	Segre and Lobotsky 1964	
4	20-22	3.3-8.2 (5.3)	0.5-3.8 (2.3)	3.5-11.7 (8.1)	3.3-27.2	Lee, 1967	

During human pregnancy all of the estrogens increase rapidly in the urine (Turner, 1963). Just before parturition estrone and estradiol increase a hundred fold and estriol, a thousand fold. Estrone and 17β-estradiol are usually excreted in a constant ratio of about 3:1. The urinary estrogens diminish rapidly after parturition and the loss of the placenta.

Healthy males in the third and fourth decades of life excrete on the average of 2, 6 and 6 μg of 17β-estradiol, estrone and estriol, per 24 hours. These values fall within the range observed during the first week of the menstrual cycle, and are approximately twice those observed in the post-menopausal or ovariectomized female. Castration appears to have only a slight lowering effect, indicating that extratesticular tissues are the chief sources of these materials (Danowski, 1962). An eight to ten day cycle of urinary estrone for normal men was reported by Exley and Corker (1966)

Influence of Estrogens on Serum Lipids

During the last 20 years the attention of many workers has been directed towards the investigation of lipid metabolism in coronary disease. Elevation of the plasma lipids has frequently been observed in patients with coronary artery disease when compared with the normal.

The clinical recognition of atherotic heart disease in the female has shown a lower incidence as compared with the male. Adlersberg et al. (1956) reported that the period of marked increase of serum lipid levels was similar for men and women except the onset for men starts 13 years earlier than in women. Russ, Eder and Barr (1955) showed that the serum of young women had

less β-lipoprotein and relatively more α-lipoprotein than that of women of older age or men of equal age. Glazier et al. (1954) noted that the mean value of the β-lipoprotein concentration in the serum was significantly higher in males than in females from the third through the fifth decade. It became approximately equal in the two sexes around the sixth decade and thereafter the mean value tended to be greater in women than in men. This correlates well with the findings of the serum cholesterol values (Hobson, Jordan and Roseman, 1953).

Following bilateral ovariectomy the degree of coronary sclerosis was more severe than in a control group of women of comparable ages (Wrest, Dry and Edwards, 1953). Following surgery the estrogen-treated group of men had less coronary heart disease than the untreated group (Higano, Robinson and Cohen, 1963).

Ritterband et al. (1963), on the other hand reported the same incidence of coronary heart disease in a group of hysterectomized women with normal ovarian function as for in a group of bilaterally ovariectomized women.

Block, Barker and Mann (1951) reported that, following a standard fat meal, men develop a significantly greater plasma lactescence than women of the same age group. They suggested that this may be of importance in the development of atherosclerosis.

In patients with hypercholesterolemia and coronary heart disease there may be a defect in the steroid hydroxylating enzymes which are responsible for the hepatic degradation of androgens. This was shown by an increase in epiandrosterone excretion in hypercholesterolemic patients whereas other

fractions were normal. Estrogen treatment of postmenopausal hypercholesterolemic women resulted in a decreased epiandrosterone excretion and a lowered serum cholesterol level (Dingman and Lim, 1963).

The ability of estrogenic hormones to alter abnormal serum lipid concentrations to a more normal pattern is well documented. They cause a rise in phospholipids without influencing cholesterol levels, resulting in a lowered plasma cholesterol to phospholipid ratio (Oliver and Boyd, 1956a, 1956b; Robinson et al., 1956; Stamler et al., 1960). Unfortunately large doses of hormone produce undesirable side effects in the male subjects who have received this treatment (Kroman et al., 1966).

Chabonian (1966) reported on the effect of estrogens on the invitro synthesis of lipids by arterial intimal tissues in dogs. Estrogens stimulated intimal phespholipids synthesis which was accounted for by an increase in lecithin and cephalin production. No effect of estrogen on arterial synthesis of other lipids was demonstrable. The studies suggest that estrogenic hormones have a direct stimulating effect upon phospholipid synthesis within the arterial intima. Although coronary atherosclerosis was inhibited by estrogens, lesions in the arota were unaffected (Pick et al., 1951; 1952a). Pick et al. (1952b), also demonstrated that estrogens were effective therapeutically in reversing corony atherosclerosis induced in cockerels by cholesterol feeding.

Albers and Riggi (1966) studied the mechanism of estrogen-induced hypocholesterolemia in the rats. It has been ascribed to either (1) stimulation of phagocytic activity of the reticulo-endothelial system, or (2) decreased hepatic sterol biosynthesis, and/or (3) enhanced fecal steroid excretion. Their data

reveal that during the development of hypocholesterolemia in estrone-treated rats (1) reticulo-endothelial phagocytic activity was unaltered, (2) sterol biosynthesis was unchanged, and (3) fecal steroid excretion was not enhanced. These studies suggest that the hypocholesterolemia is due to enhanced hepatic sterol sequestration.

Spontaneous atherosclerosis in the white correan pigeon was decreased by the administration of estrogen (Souadjian, Kottke, and Titus, 1966).

Estradiol was found to exert a potent though indirect, effect on lipid synthesis and mobilization in adult tissues of male adult rats (Steinberg and Shapiro, 1966).

There have been few studies of the blood lipids in relation to the menstrual cycle. Oliver and Boyd (1953) summarized the early studies. Conalons in 1916, found an elevated total cholested level before and during menstruation, followed by a sharp drop after menstruation. Moynihan in 1925 showed a rise in blood cholesterol before, and a fall during, menstruation. Okey and Boyden in 1927 found an elevated total cholesterol before and after menstruation, with a fall during menstruation. These workers found no variation in plasma lecithin during the menstrual cycle. Offenkrantz in 1938 estimated free and total plasma cholesterol and observed a rise in ester cholesterol during menstruation followed by a fall post-menstrually. The free cholesterol appeared to rise before, and fall during, menstruation.

Oliver and Boyd (1953) reported that identical cyclical changes have been observed in the plasma lipids in women. There is a striking fall in plasma ester cholesterol, a less marked fall in plasma phospholipids, and, therefore, a

decrease in the plasma total cholesterol: phospholipid ratio at the time of ovulation and immediately prior to menstruation. It is suggested that the point where the plasma total-cholesterol:phospholipid ratio is lowest coincides with the phase in the menstrual cycle where estrogenic activity is maximal.

Kroman et al. (1966) reported that a relationship appears to exist between the blood lipids and estrogenic hormones in control males and females and similar groups of patients having confirmed coronary artery disease. In general the control group of individuals exhibited a greater concentration of 17β-estradiol than estrone. In those individuals with coronary artery disease the converse was true. However, those individuals of the control group with the highest concentration of 17β-estradiol did not have the lowest lipid levels. On the other hand, several patients with coronary artery disease exhibited the normal levels of serum cholesterol and/or triglyceride and showed the same predominance of estrone in the plasma.

A study on four young women by Sang (1967) in this laboratory showed that the fatty acid patterns of serum lipids were influenced by the menstrual cycle, although the differences were not significant. In the cholesterol ester fraction, oleic acid predominated at the beginning of the cycle; myristic, palmitic and stearic at mid-cycle; and linoleic at the end of the cycle. Triglycerides were high in palmitic and linoleic at the beginning; stearic at mid-cycle; and oleic at the end of the cycle. The phospholipids were high in palmitic at the beginning; oleic and linoleic at the mid-cycle; stearic at the end of the cycle. The implications or causes of these changes were not investigated.

There is a possible relationship of these findings to the estrogenic hormonal influence on lipid metabolism and indirectly to the well known sex difference in coronary disease.

Diet and Serum Lipids

It is well known that multiple nutritional factors including total calories, fat, carbohydrates, proteins, minerals, and vitamins influence the level of the blood lipids.

According to Friedman's (1968) recent review, Keys et al. in 1950 and 1955, showed that there were significant decreases in total cholesterol in young volunteers on a low-calorie diet. Kartin et al. (1944), reported a significant increase in cholesterol, larger rises in phospholipids and critical changes in triglycerides in man during total starvation. These changes were reversed by the ingestion of carbohydrates. Friedman (1968) also mentioned that both the fat-and carbohydrate-induced hypertriglyceridemias responded to low calorie intake.

The effect of excess calories on serum lipid levels has not been well defined. A group of investigators found that an excess of calories over a short period of time caused a striking increase in serum cholesterol. The hypercholesterolemia reached its peak in a few weeks and was maintained unchanged, despite a continuing gain in weight. These studies did not show differences in the blood serum of overweight individuals as compared to normal or underweight individuals (Friedman, 1968). The observations of many investigators clearly indicated that the intake of excess calories in normal, as well as in hyperglyceridemic

patients, results in an elevation of the serum triglycerides (Kuo and Bassett, 1963). Moreover, excess body fat imposes an increased workload on the heart and thus increases the strain on the circulatory system.

Efforts have been made to relate differences in blood lipid levels to various dietary habits. McGandy, Hegsted and Stare (1967) in a recent review noted the difficulty of ascribing population differences in blood lipids to dietary practices alone, even in the careful international epidemiologic studies of Keys et al. (1966) and Keys (1970). The lack of success in relating an individual's level of serum cholesterol to his dietary practices has also been reported (Browe et al., 1967; Paul et al., 1963). However, studies in man under controlled dietary conditions should help explain the influences of various dietary factors on serum lipid levels. The importance of dietary fat has attracted major attention in this field.

The intake of total fat exerts a strong influence on serum lipid levels. Populations of people with diets high in total fat usually have high serum cholesterol levels. Those with a low intake of total fats usually have low cholesterol levels. However, there is considerable individual variation in the level of serum cholesterol among those with the same total fat intake. Hegsted et al. (1967) reported marked variations in the serum cholesterol of individuals consuming a constant diet. Although the serum cholesterol levels were changed by alterations in the diet, the person to person variability persisted.

Elevations of serum triglycerides have been reported with diets low in total fat and high in carbohydrate (Albrink, 1962; and Bierman and Hamlin, 1961). However, this hypertriglyceridemia is transient, as shown by Antonis and

Bersoln (1962a and 1962b). In a carefully controlled study on South African white Bantu prisoners, they showed that a diet low in total fat and high in carbohydrates, when followed for a period of 39 weeks, caused a lowering of all serum lipids, including cholesterol and triglycerides. This finding was true for both European and Bantu groups when compared to the usual low levels found in Bantu natives. The diet was then changed to one containing less carbohydrate, more fat and the same amount of protein. Those who received the high fat intake in the form of polyunsaturated fat, maintained the same low lipid levels, while those who received the fat in the form of hydrogenated oil, or butter, developed high lipid levels. In the final period, where all subjects were given the original diet, low in fat and high in carbohydrate, the serum cholesterol fell rapidly, but the serum triglycerides rose rapidly in all. The levels of serum triglycerides then fell gradually to the low levels usually found in those who habitually consume a low fat, high-carbohydrate diet.

It is well known that the quality of dietary fat also exerts an influence on the levels of serum lipids. In a recent review, Friedman (1968) indicated that diets high in vegetable fats caused a marked decrease in the serum cholesterol and phospholipid levels, while the substitution of isocaloric amounts of animal fats caused a rise in these levels. However, not all vegetable oils lowered serum cholesterol, and not all animal fats raised it.

It has become apparent that changes in serum lipids and cholesterol are dependent on the degree of saturation or unsaturation of the fats, rather than on their source (Keys and Blackburn, 1963). Highly unsaturated oils decreased the

levels of serum cholesterol, while iso-caloric amounts of highly saturated fats increased the levels.

Most animal fats are rich in saturated fatty acids, but some have a high degree of unsaturation. Most vegetable oils are rich in unsaturated fatty acids with exceptions of coconut oil and olive oil. The composition of the animal fat varies with the animal's diet and with the season of the year; that of vegetable oils varies with the variety of the product and the methods of its production. Thus, the arbitrary division of fats into animal and vegetable is chemically meaningless.

The essential unsaturated fatty acids (EUFA), linoleic, linolenic and arachidonic, which are present in most oils, but not butter, exert a lowering effect on the serum cholesterol. When the polyunsaturated fatty acids were hydrogenated, their favorable effect on serum cholesterol was lost, then they acted to elevate it just as did certain highly saturated fats such as butter and coconut oil. This observation has been confirmed by others (Connor et al., 1969; Grundy and Ahrens, 1966; Grande, Anderson, and Keys, 1965; Moore et al., 1968; Wood, Shioda and Kinsell, 1966).

The saturated fatty acid responsible for the elevation of serum cholesterol has been reported to the palmitic acid (Keys, Anderson and Grande, 1965c) or myristic acid (Stare, 1966). Depot fat analysis of subjects on a low saturated, high polyunsaturated, fat diet for periods of one to four years have revealed lowering of both palmitic and myristic acids with a concomitant rise in linoleic acid (Christakis et al., 1965). The type and amount of fat in the diet influenced the composition among individual cholesterol esters (Nestel

et al., 1965, 1966). The composition of serum cholesterol esters was only minimally affected by a single meal of a specific fat (Kayden, Karmen and Dumont, 1963) but was readily influenced thereafter. The changes in cholesterol ester composition were apparent within 24 hours after diets were changed and were evident in all classes of lipoproteins (Nestel and Couzens, 1966). The substitution of a diet rich in saturated fats for one rich in carbohydrates interestingly led to an increase in the proportion of cholesterol linoleate and a fall in the proportions of saturated and monounsaturated fats although the intake of linoleate remained low. This suggested that saturated and unsaturated fatty acids were incorporated into serum cholesterol esters more readily than they were derived from endogenous synthesis than from the diet (Nestel and Couzens, 1966). The method of cooking and eating might influence the serum lipid levels by causing changes in the size of the fat globules (Malhotra, 1967).

The serum cholesterol concentration was also influenced by dietary cholesterol (Connor et al., 1961, 1964; Erickson et al., 1964). The daily intake of cholesterol in the human varied from 200 to 800 mg (Friedman, 1968) and from 500 to 2,000 mg in the Western World (Keys, Anderson and Grande, 1965) and was highest in those with a high intake of saturated fat. The body synthesizes approximately 2,000 mg daily from various tissues (Friedman, 1968). In man no more than 40 per cent of circulating cholesterol was derived from the diet even when high-cholesterol diets were consumed (Grundy and Ahrens, 1969; Kaplan, Cox and Taylor, 1963; Wilson and Lindsey, 1965). Although the absorption of dietary cholesterol is relatively limited in man, when acute single loads

of cholesterol were fed a much higher amount could be absorbed (Borgström, 1969). With prolonged repeated ingestion of high-cholesterol diets, absorption declined (Grundy, Ahrens and Davigon, 1969).

Recent studies in man comparing cholesterol balance measurements by gas-liquid chromatography with isotopic balance and specific activity time curves reveal new insights into cholesterol metabolism (Nestel, 1970). Dietary cholesterol apparently provides no feedback control over cholesterol synthesis, although sequestion of endogenous cholesterol can stimulate it. The concentrations of cholesterol in serum did not necessarily reflect cholesterol synthesis in man (Goodman and Noble, 1968; Nestel, Whyte and Goodman, 1969; Wilson and Lindsey, 1965). In a recent report on quantitative relationships between dietary lipids and human serum cholesterol concentrations, Key's group (Fetcher, et al., 1967) noted that dietary cholesterol raises the serum cholesterol in proportion to the square root of its concentrations in the diet. Other studies (Connor, Stone and Hodges, 1964; Hegsted et al., 1965) have suggested a direct relationship between the dietary cholesterol and the serum lipid levels. Restriction of dietary cholesterol has been recommended as helpful in reducing serum cholesterol. It has been suggested that the omission of egg yolk from the diet is about the only food that will reduce cholesterol levels. To include enough polyunsaturated fatty acids to have much effect on the serum cholesterol requires changing food habits drastically (Christensen, 1967).

Yudikin and Roddy (1964) and Yudikin (1967) had stressed the importance of the role of carbohydrates on serum lipid levels. The serum cholesterol or all blood lipid levels were lower on diets containing complex sugars in the form

of starch from cereals and potatoes than those containing simple sugars (Antar and Ohlson, 1965; Irwin, Taylor and Feeley, 1964; Keys, Anderson and Grande, 1960). This applied regardless of whether the fat content of the diet was low or high (Keys, Anderson and Grande, 1960).

McGandy et al. (1966) reported lower serum cholesterol levels on diets with low sugar content. However, the changes induced by dietary fats were much greater and were independent of the kind of carbohydrate, although controlled studies have demonstrated slight reductions in blood lipids when simple sugars are replaced by complex carbohydrates in the diet. These changes are of a small order as compared with those obtained by changes in fats (McGandy, Hegsted, and Stare, 1967). No differences related to the type of simple sugars (glucose, sucrose or lactose) have been reported except by McGandy et al. (1966) who noted an enhancement, by dietary lactose, of the hypercholesterolemia caused by a high saturated fat intake.

Grande, Anderson and Keys (1965) reported an 18 per cent decrease in cholesterol on substituting carbohydrate from legumes for the sucrose, although no significant change in serum cholesterol occurred when bread and potatoes were substituted for sucrose. They concluded that either the carbohydrate or some other substance, in leguminous vegetables led to the cholesterol reduction.

A high-carbohydrate low-fat diet has resulted in hypertriglyceridemia in normal (Antonis and Bersohn, 1961; Herman, Zakim and Stifel, 1970); hypercholesterolemic (Kuo and Bassette, 1963) and hypertriglyceridemic (Ahrens et al., 1961; Fredrickson, Levy and Lees, 1967) patients. MacDonald and Braithwaite (1964) reported an elevation of triglyceride alone on a sucrose diet.

This did not occur when starch was substituted for sucrose. Increased glucose or sucrose content caused the serum triglycerides to increase, but sucrose alone always caused a greater increase than the corresponding percentage of glucose. No significant changes occurred in serum cholesterol or phospholipid and body weight changes were minimal (Herman, Zakim and Stifel, 1970).

In 1965 MacDonald reported further studies using semipurified formula diets essentially free of exogenous fat. The results were different for male and female subjects. The lipid pattern of the men on diets high in sucrose became similar to those of men with coronary heart disease, which was quite in contrast to that of young women.

The effect of the quantity and quality of carbohydrate on serum lipid levels must be interpreted with caution in all short term feeding experiments in view of the work of Antonis and Bersohn (1961, 1962a, 1962b). Alterations in the source of dietary carbohydrates might not be a useful addition to modifications of dietary fats in achieving blood cholesterol reductions (McGandy, Hegsted and Stare, 1967).

The mechanism whereby dietary carbohydrate leads to markedly elevated serum triglycerides is not known. Some individuals have high serum triglyceride concentrations which can be markedly decreased by removal of dietary carbohydrates from their diet or by drugs. Others fail to respond to dietary change or drugs for reasons yet to be determined. Above normal activity of glycolytic and lipogenic enzymes may be responsible for rapid conversion in the liver of dietary carbohydrates to triglycerides which spill over into the blood (Anonymous, 1971).

Experimentally, the effect of dietary protein levels on serum lipids in man and animals has given conflicting results. A deficiency of labile methyl groups in the diet of rats resulted in hypocholesterolemia, even when the diet also contained cholesterol (Wilgrom, Lewis, and Blumenstein, 1955). The hypocholesterolemia reported by Olson, Jablonski and Taylor (1958) was not affected by the quality or quantity of dietary fats. The decreased ability for choline synthesis was suggested by Ahrens (1957) as being responsible for decreased serum cholesterol levels.

It has also been shown that the feeding of proteins deficient in sulfur leads to hypercholesterolemia in cholesterol-fed monkeys (Mann et al., 1953). Portman and Mann (1955) demonstrated that this type of sulfur deficiency inhibited the production of taurine and hence of taurine-conjugated bile acids. With the conversion of cholesterol to taurine-conjugated bile acids being limited by sulfur deficiency in the diet, thus, cholesterol accumulates in the plasma. Morse et al. (1966) also noted that sulfur-containing amino acids might indirecly affect serum cholesterol levels.

Keys and Anderson (1957) showed that varying the protein intake from 11-20 per cent of the total calorie intake had no significant effect on the serum cholesterol level in man. Similar results are also reported by Wilcox, Galloway and Taylor (1964). Their findings indicated that serum lipid levels were not affected by milk (O, 1 or 2 quarts) or dietary protein intake (10, 13.4 or 16.8 per cent of calories) in University athletes. Olson et al. (1958) suggested that the level of protein intake may be an important determination of the serum lipid levels in man. The feeding of 25 g vegetable-cereal protein with iso-calorie

and fat resulted in a significant drop (20 per cent) in serum cholesterol with a lesser drop in phospholipid. Ultracentrifugal measurement showed that this decrease in serum lipid was due primarily to a decrease in the S_f[1] 0-12 β-lipoprotein fraction. They suggested that an amino acid imbalance in which methionine played a role was responsible for the serum lipid-lowering effect. A decrease was observed in serum cholesterol levels in man or woman by replacing animal protein with vegetable protein in a diet with or without cholesterol (Hodges et al., 1967; Walker, Morse and Overley, 1960). The same was true for studies in rats (Nath, Harper and Elvehjem, 1958; Olson, Jablonski and Taylor, 1958). Olson (1967) and Olson et al. (1961, 1964,and 1970) recently showed that amino acids other than metionine were also involved in the hypocholesterolemic effect.

It was noted that the hypolipemia of kwashiorkor can be corrected by the administration of fat-free milk powder (Frenk et al., 1958; Scrimshaw et al., 1956). An increase in the serum cholesterol concentration in children fed diets containing high levels of either animal or vegetable proteins was observed by Bagchi, Ray and Datta (1963). On the other hand, Beveridge and his coworkers (1963) indicated that purified proteins in the absence of variation in dietary fats and cholesterol had no such effect. The latter experiments were conducted during a brief 8-day period for each dietary regimen. Rosenfeld and Lang (1957) reported that reducing dietary protein from 19 to 6 per cent of total calories caused a decrease in serum phospholipid in the rat, but a recent study (Patel, 1969) in this laboratory showed that dietary intake of protein did not

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[1]Svedberg flotation unit an expression of flotation velocity of the plasma proteins, (See Friedman, 1968, p. 887 and Masoro, 1968, pp. 181-182).

significantly affect serum lipid levels, unless fed at extremely high levels (50 per cent of weight of diet). The proportion of oleic acid in liver phospholipids and triglycerides decreased in the rat.

A recent report by Tripathy, Lotero and Bolaños (1970) showed a highly significant increment of serum cholesterol and phospholipid levels by protein repletion (35 to 100 g) in malnourished adults with a variation in dietary fats and cholesterol. Surprisingly, Elson, Humleker and Pascal (1971) observed that lower serum cholesterol and higher phospholipid levels when subjects were fed a low protein (48 g) diet with the comparison of a high protein (141 g) diet in man. Friedman (1968) stated that the amount of protein in the diet, if adequate, does not influence the serum cholesterol. A fall in serum cholesterol, plus β-lipoproteins, will occur when the intake of protein is reduced to deficiency levels.

It is well known that nicotinic acid in large doses, lowers the serum cholesterol. The mechanism by which nicotinic acid reduces the serum cholesterol is only partially understood. It is thought to have an effect on the enzyme systems of the liver which are involved in cholesterol synthesis and their conversion to bile acids (Parsons, 1961).

A correlation of the serum magnesium in man (Bersohn and Oelofse, 1957) showed that the higher the serum cholesterol level, the lower the serum magnesium. Administration of enteric-coated magnesium chloride has failed to alter serum lipids significantly in human subjects (Haywood and Selvester, 1962).

Regardless of the gaps that remain in our present day knowledge of the effect of diet on serum lipids, it seems clear that the responses of the serum lipids to dietary changes is due almost entirely to the effects of dietary fats.

The effect of the type of carbohydrate is dependent upon the level and degree of saturation of the fat content of the diet. The total calories, total fat intake, degree of saturation and chain length of the fatty acids, and to a lesser extent quantity of cholesterol in the diet are the major dietary factors in the control of the serum lipids and lipoproteins.

Lipids and Atherogenesis

Atherosclerosis is the main cause of death in those countries which are highly developed industrially and which enjoy a high standard of living. It has been characterized by the variable combination of changes in the intima of arteries, consisting of focal accumulation of lipids, complex carbohydrates, fibrous tissue and calcium deposits. It will be noted that lipids are only one of several factors involved in this disease.

Atherosclerosis affects the large and medium sized blood vessels, such as the aorta, coronary, cerebral, renal, iliac and femoral arteries, producing its deleterious effects in one of two ways; either by a gradual narrowing of the artery leading to a reduction in blood supply to the affected part or by sudden occlusion of a blood vessel, due to a thrombus being superimposed on an atherosclerotic lesion (Lawrie, 1963). It is felt that the lesion starts as a fatty streaking of the intima, with most of the lipids being present in lipid-infiltrated cells which lie immediately beneath the endothelium; and that these lipid streaks become smooth, yellow or white, button-like plaques. At this stage, part of the lipid is within lipid-infiltration cells and part forms a structureless lipid mass outside the cells (Portman, 1970). However, whether or not the

plaques come from the fatty streaking in the manner described, although plausible, is far from proved.

The etiology of atherosclerosis is, at present, imperfectly understood. The high lipid content of atheromatous plaques supports the hypothesis that lipids play a role in the pathogenesis of atherosclerosis. The exact mechanism or mechanisms by which lipid is deposited in the vessel wall is still unknown. Many theories have been postulated. Some workers picture an initial fatty infiltration, others an initial arterial damage followed by fatty infiltration, and still others believe that the metabolic role of the tissue is also a contributing factor (Friedman, 1968; Kritchevsky, 1962; Lawrie, 1963; Portman, 1970). Whatever the final definitive elucidation of the pathogensis of this disease, it is now one of the most pressing medical problems of the Western societies.

Cholesterol ester is the major class of lipid that accumulates; triglycerides, phosphoglycerides, sphingolipids and free cholesterol are present in small amounts (Portman, 1970). The cholesterol ester fatty acids are palmitic, stearic, oleic, linoleic, arachidonic and palmitoleic penta-and hexaenoic acids. The data of cholesterol ester compositions differ among themselves in some aspects but in general there is over-all agreement (Kritchevsky, 1962; Portman, 1970). There has been no rigid proof that reduction of serum cholesterol levels in man will decrease the incidence of coronary disease; although available data has shown that cholesterol is laid down in plaques, some did turn over and some regression did occur in animal experiments (Friedman, 1968).

Although in man atherosclerosis is an inevitable concomitant of aging, in diseases such as diabetes, hypothyroidism, myxedema, nephrosis and

xanthomitosis, all exhibit markedly increased levels of serum cholesterol. The rate and extent of atheroma production are also elevated (Adams, 1967; Kritchevsky, 1962). In the Framingham and other studies (Kannel et al., 1966; Albrink, 1965), it was found that plasma cholesterol and triglyceride levels are positively correlated with coronary artery disease. A serum cholesterol level above 260 mg per cent is associated with an increased risk of coronary artery disease. In populations where coronary artery disease was rare in middle-aged men, the mean serum cholesterol levels were in the range of 130 to 170 mg per cent. This plus other data based on the cholesterol levels in those who have had a definite myocardial infarction, the use of values in young adults as normal values, as well as data on long term prospective epidemicological studies, suggested that the present normal cholesterol value of 250 mg per cent is too high. An appropriate level for "normal", based on the vast amount of information available is approximately 200 mg per cent. Therefore, it must be concluded that a large majority of middle-aged Americans are hypercholesterolemic (Stamler et al., 1966).

Albrink (1965) postulated that a higher concentration of serum cholesterol would be innocuous if triglycerides were normal, since the physical state of the cholesterol is dependant on the level of the triglycerides.

In all subjects with elevated serum lipid levels, regardless of degree or type, every reasonable means available should be used to control the lipemia. The lowest possible serum lipid levels should be sought in the prophylaxis of coronary disease (Furman, 1967).

Since coronary artery disease correlates with atherosclerosis, these serum lipid levels are also correlated with atherogensis. That the correlation of coronary artery disease with serum triglyceride levels is as good as its correlation with serum cholesterol levels possibly relates to the fact that hypercholesterolemia is usually accompanied by a hypertriglyceridemia. This correlation between serum lipid levels and atherogenesis has led to the widespread use of dietary therapies aimed at controlling blood cholesterol to triglyceride levels. This subject has been previously reviewed.

Although there is evidence that cholesterol and possibly triglycerides are in some way linked to the atherogenic process, whether they are the primary etiologic agents or the secondary influence on the systems is not known, nor is there information regarding the precise mechanism by which lipids accumulate in atheromatous lesions. No definite conclusion can be drawn for the role of lipids played in atherogenesis.

METHODS AND PROCEDURES

Statement of the Problem

The purpose of this study was to investigate the levels and monthly variations of serum lipids and urinary estrogens in healthy menstruating young women under normal living conditions. The serum cholesterol, phospholipid (lipid phosphorus), triglyceride, total lipid values and fatty acid patterns of three lipid fractions (cholesterol, phospholipid and triglyceride), and urinary estrone, estriol and 17 β -estradiol levels were determined.

Description of the Subjects

Fifteen female university students served as experimental subjects. Originally, twenty or more individuals were sought, but difficulty was encountered in obtaining an adequate number of volunteers to join the program.

Except for three persons who were eliminated for medical reasons, all of the initial participants continued to the termination of the study. The age range of the twelve participants was 18 to 23 years, with a mean of 20, except for one subject who was 40. Data on age, height and weight are presented in Table 5.

The study was conducted from September 1968 to May 1969, with each subject participating for four to five weeks.

All the subjects received a physical examination by the University Health Service[1] before starting the experiment and were found to be in normal condition.

[1]Dr. Willis Hayward

Table 5. Data on age, height and weight of subjects

Subject	Age	Height		Weight
		Ft.	In.	lbs.
AM	20	5	1.5	130
CO	18	5	4.5	130
CP	18	5	7.0	123
HL	40	5	5.0	165
JB	20	5	1.5	111
JE	22	5	6.5	144
LR	23	5	4.0	125
MA	20	5	6.5	120
MC	19	5	9.0	145
MH	20	5	6.5	125
RM	19	5	5.0	125
SL	21	5	5.0	125

Laboratory examinations were performed in the Nutrition Research Laboratory. The enumeration of red and white cell counts was by Wintrobe's Standard Clinical Procedures. Values were found within normal ranges cited by Faulkner, King and Damm (1968) and Wintrobe (1961).

Although the subjects were in general good health, organic disorders were reported by three persons: AM was allergic to aspirin; HL, penicillin; JB, a skin allergy which was controlled by limiting certain kinds of foods. Subjects LR and MA had taken birth control pills for six months to one year, until a year before the study was started.

After blood was taken, the subjects weighed themselves and recorded their weight. Then, breakfast was served to the subjects in the Nutrition Department. Subjects were asked to maintain constant weight on their self-selected diets.

Specific instructions (Wilcox, Gillum and Hard, 1956) were given to the subjects to record their food intake for two days a week on forms provided (Appendix B). Food record of the day before urine collection was preferred.

Dietary intakes were calculated. Calculations were made for: the content of calories, protein, fat, carbohydrate, saturated and unsaturated fatty acids (oleic and linoleic) and cholesterol. The percentages of total calories were also calculated. Calculations were made from food composition tables from the following sources: Browe et al., 1966a, 1966b; Church and Church, 1963; Krause, 1961; Leverton and Odell, 1958; Watt and Merrill, 1963.

Collection and Preparation of Blood Serum

The subjects reported to the laboratory before breakfast on certain days during a complete menstrual cycle. Ten ml of blood was taken[1] from the antecubital vein on the following days: the beginning of menstruation (day 1 or 2), the end of menses, midcycle (days 12 to 16), third week (days 22 to 24), and before the onset of next menstruation. The drawn blood was allowed to clot at room temperature for at least a half hour and then centrifuged at 2500 rpm for 30 minutes. Blood serum was transferred to the appropriate size tube, and stoppered with rubber stoppers. The tubes were kept at -10 C until analyses were made.

To eliminate possible day to day variations in the quantitative analyses, most of the studies were performed by making a lipid extract of the serum immediately after the sample was taken and storing this extract in an airtight flask in a cold (-20C) dark place. When the samples had been obtained from each individual, all the estimations were conducted on the same day.

Collection and Storage of Urine Specimens

Instructions for collecting 24-hour urine specimens were given to each subject, that is, to discard the first morning specimen on the day of collection and to collect all urine voided up to and including next day's first urine specimen. Containers of adequate size (two one-quart bottles) labeled with name and date were given to the subjects for the collection.

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[1] Mrs. Sue Do, Medical Technician.

Creatinine values were used to check the accuracy of the completeness of the total urine for the 24-hour collection since these values have been shown to be nearly constant for a given individual. The standard clinical method (Bauer, Toro and Ackermann, 1962), was used to determine the urinary creatinine levels of each 24-hour collection.

A 24-hour urine sample was collected daily at mid-cycle (day 12 to 16) and the third week (day 21 to 24) and every third day for the rest of the cycle (day 1, 4, 7, 10, 19 and 27) for one complete cycle.

Although all of the urinary steroids are relatively stable compounds, a preservative was added to each bottle in which urine was to be collected. A mixture of penicillin and streptomycin (1,000 units of penicillin and 5 mg of streptomycin for each 24-hour collection) was selected as the preservative to prevent both bacterial contamination and deterioration upon frozen storage. All the specimens were made up to constant volume were then stored at -10 C until estrogen analyses were made.

The subjects were instructed in the use of a clinical thermometer, and asked to record their waking oral temperature every morning while still lying in bed. Ovulation was judged to occur at or just before the point where the temperature began to rise during the intermenstrual phase (Oliver and Boyd, 1953).

Methodology Studies of Serum Lipids Analyses

During the last decades, methodological advances in the lipid field arose mainly from the introduction of chromatographic techniques. The combination of these with known procedures led to improvements on considerably smaller

amounts of materials, with greater efficiency and rapidity. It also increases precision of data on the individual components under the investigation. In the following determinations, emphasis is laid on newer methods; older procedures which in many cases were shown to be less reliable and time consuming are not evaluated, although they have been used extensively in many laboratories.

Extraction of total lipids

Quantitative isolation of lipids from tissues and body fluids requires that the lipid-protein complexes occurring in biological materials be destroyed. For this purpose solvents have to be used which are sufficiently polar to split the complexes and denature the proteins. Complete extraction of lipids is achieved by additions of lipophilic solvents. Among the several mixtures used, chloroform-methanol and ethanol-diethylether have been very popular (Alling, Svennerholm and Tichy, 1968; Bosch and Camejo, 1967; Gjone and Orning, 1966; Wagener, 1967).

Based upon the evidence cited above, two methods were investigated for the extraction of total lipids. The method described in detail by Lis and Okey (1961), which uses an initial hot ethanol extraction, a second extraction with ethanol-diethyl ether, and a final refluxing with diethyl ether in a soxhlet, was compared with the method of Folch, Lees and Sloane-Stanley (1957) modified by Smith (1965), which uses two successive extractions with a chloroform-methanol solvent system. The method comparisons were made as to: the quantitative extraction of total lipids and the degree of degradation and the relative rates of extraction of the different lipid components with successive extraction steps.

The weights of the total lipid extracted by both methods were comparable which agreed with data reported by others (Jesting and Bang, 1963; Stevan and Lyman, 1963). However, thin-layer chromatographic analysis of the total lipid mixtures showed that some degradation occurred by the ethanol-ether extraction, but little by the chloroform-methanol extraction.

Thin-layer chromatographic analysis of the individual extracts of serum lipids obtained by both methods showed that different solvent combinations extracted certain lipid classes more readily than others. It was found that the initial ethanol extract contained all of the free cholesterol (FC) and most of the cholesterol esters (CE) and phospholipids (PL) as well as some triglycerides (TG) and free fatty acids (FFA). The ethanol-ether extraction contained some TG, FFA, and PL and a small amount of CE. After 4 hours of refluxing with ether, the extract contained TG, FFA, some PL and CE, and products that had been altered during the extraction. For lipid extraction according to Wagener (1967), heating is often employed although unnecessary for complete extraction. Moderate temperatures (30-50 C) and short heating periods should not be exceeded in order to avoid oxidative damage. In the method of Folch, Lees and Sloane-Stanley (1957) and modified by Smith (1965) all of the CE and TG and most of the PL and FC were extracted initially from the sample. The second extraction of the precipitate contained small amounts of PL and FC, whereas comparable amounts of FFA were present in both the initial and the second extraction. Several more extractions of the precipitate were tried but only traces or negligible amounts of FC and FFA were found.

From the analyses of the individual lipid extracts obtained by both methods, the effectiveness with which total lipid was extracted by either method was

not quite satisfactory. It was concluded that a selective combination with minor modifications would be most satisfactory.

The procedures adopted involved extractions with chloroform-menthol at different degrees of temperature. The modifications were based on several factors.

1. Phospholipids were more soluble in chloroform-methanol than in ethanol-ether and nearly total extraction of the PL occurred in the initial extraction. This fact confirmed the early studies of Thannhauser and Setz (1936).
2. Cold chloroform-methanol which was used in the first extraction, decreased as much as possible the exposure of lipids to heat thereby excluding oxidation of unsaturated lipids.

The procedures of total lipid extraction in detail is given in Appendix C-1.

Determination of total lipids

The gravimetric and photometric methods were suggested by Wagener (1967) for quantitative determination of total lipids. Gravimetric methods do not alter the material and so permit further procedure to be performed; the latter method is less time consuming and thus superior to older procedures.

A gravimetric measurement (Folch, Lees and Sloane-Stanley, 1957; and Smith, 1965) was investigated but was unsuccessful due to great variability.

A photometric procedure was investigated (Zöllner and Kirsch, 1962) which was based on the reaction of unsaturated fatty acids with concentrated sulfuric acid, a mixture of phosphoric acid and vanilline with development of a pink color. Since the reaction on the various lipids showed different extinction

coefficients, calibration was necessary according to gravimetric values. Control of the highly variable results by this photometric method was difficult.

Finally the colorimetric method of Bragdon (1951) was adopted. This method is based on oxidation of a suitable extract by a potassium dichromate-sulfuric acid reagent and the colorimetric determination of the reduced dichromate. The color has been shown to be stable between 15 and 90 minutes after dilution. After checking color development at six time intervals, 30 minutes was selected. The method in detail is given in Appendix C-2.

Determination of cholesterol

The estimation of cholesterol in serum is being carried out daily by most clinical chemistry laboratories at the request of physicians who are interested in using this test as an indication of atherosclerosis and as a predictor of coronary heart disease.

Numerous methods have been developed and many of those are being used today even though the method has not been standardized against a control standard of cholesterol as used in research investigations, and many of them will regularly give falsely high or fluctuating values under variable conditions. Also, many of the methods in use are not being performed properly because of lack of knowledge concerning the chemical reactions involved. The inter-and intramethod variations have been mentioned by many investigators (Kritchevsky, 1962; Tonks, 1967; Wagener, 1967). Quantitative determination of cholesterol is based on color reactions which are not absolutely specific. Contaminants present in the serum or in the reagents may seriously affect the color development. The color reactions include the Liebermann-Burchard (L-B) reaction with sulfuric acid and acetic

anhydride, the Tschugaeff reaction with zinc choloride and acetyl chloride in acetic acid, or the Kiliani (Lifschütz) reaction with ferric chloride in acetic acid and sulfuric acid. Both free and esterified cholesterol participates in these reactions. Methods or modifications of the methods which are commonly used are those of Schoenheimer and Sperry (1934), Sperry and Webb (1950), Zak et al. (1954), Zlatkis, Zak and Boyle (1953). They were recently reviewed critically by Vanzetti (1964) and Tonks (1967).

Three different methods for the determination of serum cholesterol were investigated. The methods included: saponification of esters and precipitation with digitonin according to the procedure of Galloway et al. (1957). It was based on techniques of Sperry and Webb (1950); fluorimetric method following the procedure of Tishler and Bathish (1965), an ultramicromethod which was based on the procedures of Albers and Lowry (1955); and a rapid method outlined by Clark, Rubin and Arthur (1968) which was a shorter procedure of the Mann (1961) method that eliminated the saponification, extraction and evaporation steps.

Although the method of Galloway, et al. (1957) for the determination of cholesterol had been used extensively in this laboratory, it was time-consuming and required proficiency with rigid control of conditions in the highly standardized technique to obtain results with accuracy and precision. The Liebermann-Burchard reaction was complex, and could be affected by many variables, such as the concentrations of each component, the amount of water (if any) in the final reaction mixture, the solvent employed, the time of reaction, the temperature of reaction, light, the wavelength at which the color is measured, the presence of interfering substances such as bilirubin and unreacted digitonin and the form in

which the cholesterol is present, that is, esters, free or both. Therefore, the conditions must be rigidly controlled. Also, from a technical point of view, the instability of the reagent itself and the instability of the final color were particularly annoying. This necessitated the preparation of reagent just before addition and the use of a carefully timed and planned sequence for the addition of the reagent to a series of tubes. Many of the variables of the L-B reaction have been discussed thoroughly by Martensson (1963). The Tishler and Bathish (1965) method is based on the Tschugaeff reaction. Although the procedure is applicable to the analysis of as little as 2 μl of serum, difficulty in its use was encountered. The 20 minutes incubation at 65 C caused evaporation of the highly volatile acetyl chloride.

In 1968, Clark, Rubin, and Arthur published a method which utilized iron salt-sulfuric acid reagents. It is based on the procedures of Abell et al. (1952) and the Kiliani, $FeCl_3$-H_2SO_4 (FeSac) color reaction described by Zlatkis et al. (1953). It is similar to the Mann (1961) method. It was developed principally for determining total cholesterol in small amounts of serum (0.05 ml) and for rapid analysis of many samples while maintaining the accuracy and precision of lengthier procedures. A modified iron reagent, ferric chloride in ethanol (FeAlc), was added first to the serum then alcholic potassium hydroxide (KOH-ethanol) followed and incubation at 65C for 5 minutes to aid protein-$Fe(OH)_3$ precipitation. After the removal of protein the iron-ethanol reagents and sulfuric acid were added separately, the iron-cholesterol chromogen was extracted by methylene chloride and the optical densities were read at 525 mμ.

The comparison of methods was done to determine the accuracy of this method. The method of Abell et al. (1952) was used as the reference method. Ten serums were analyzed by these two and the Mann method. There was good agreement between the values for each serum sample and also with data reported by Mann (1961) and Clark, Rubin, and Arthur (1968).

It has been shown conclusively by Webster (1963) that a ferric chloride reagent followed by sulfuric acid separately gives the same intensity of color with cholesterol from serum regardless of whether it is in the free form or is esterified. Therefore, saponification is not necessary when using the Kiliani reaction which makes the method simpler than methods using the L-B reagent systems. A high sensitivity of the Fe^{+++} - H_2SO_4 reagents makes this reaction ideally suited for tests with micro and ultramicro samples. The color is also sufficiently stable to enable the anlyst to read several samples in sequence without the exact timing necessary with methods using the L-B reaction. In addition, the color is not sensitive to light. While evaluating this method, it was found that most of the interfering substances in serum, such as bilirubin and bromide, can be eliminated by the use of ethanol to extract serum cholesterol, and that variations due to impurities in the acetic acid can be avoided by the use of ethanol as the solvent for ferric chloride.

These two possible sources of interference to the iron salt-sulfuric acid methods have been reported by Martinek (1965) and Tonks (1967). This method has all the advantages cited above. However, a rapid standardized technique of sulfuric acid addition and mixing is essential for reproducible color formation

since the maximum formation of the iron-cholesterol chromogen is dependent on exceeding a critical temperature during mixing of the color reaction reagents.

Although the determination of the esterified cholesterol has been used as an index of liver function, recent studies have shown that measurement of the esters contributes scant clinical information (Franey and Amador, 1968). Hence, no attempt was made for the determination of either esterified or free cholesterol in this study.

It was found unsuccessful (by this method) to determine the esterified cholesterol by digitonin precipitation of free cholesterol from an aliquot of supernatant. However, the authors have claimed that the determination of esterified and free cholesterol may be done.

The Clark, Rubin, and Arthur (1968) procedures were adopted for this study. It is described in detail in Appendix C-3.

Determination of phospholipids

Total phospholipids are quantitated by determination of the phosphorus content of lipid extracts from which non-lipid phosphorus has been removed by purification procedures. For this purpose chloroform-methanol extracts subjected to diffusion purification (Folch et al., 1951; Folch, Lees and Sloane-Stanley, 1957; Sperry, 1955) are suitable which has been previously discussed in total lipid extraction section on pages 37-39. Lipid phosphorus is determined in aliquots of these extracts after digestion which converts the organic phosphorus compounds to inorganic phosphate. Most procedures for determination of phosphorus are based on the method of Fiske and Subbarow (1925) which utilizes conversion of phosphate to phosphomolybdate and its subsequent reduction to

molybdic blue. Modifications of this method were reviewed by Lindberg and Ernster (1956). Lately, further modifications of this method have been proposed for the purpose of increasing sensitivity, reproducibility and accuracy (Bartlett, 1959; Draml, 1966; Shin, 1962). In several methods, the colored heteropoly complex is extracted from its aqueous environment-either to eliminate serious error caused by labile organophosphate compounds in the analyzed material or to increase sensitivity (Hurst, 1964; Penniall, 1966; Rhodes, 1955). In general, digestion involves boiling the material (Baginski and Zak, 1960; Baginski, Fao, and Zak, 1967a, 1967b). Total phospholipids are calculated by multiplication of the lipid phosphorus values with 25. These values are only approximations since phosphorus does not represent exactly 4 per cent of each phospholipid molecule.

Two methods were investigated: The methods included acid-oxidizing mixture incineration following the procedure of Lowry et al. (1954) and acid-calcium digestion according to the procedure of Baginski, Fao and Zak (1967a) and Baginski et al. (1968).

Actually both methods were based on the same principle that phosphate was converted into phosphomolybdate complex and reduced by ascorbic acid, but the former method had a time consuming series of steps; a 2-hour period for both drying and ashing, in addition to the 2 hours for incubation, as well as other disadvantages that have been reported. It may lead to a loss of phosphate or its rearrangement to a nonreactive form (Glick, 1963); the strong acid residue interfered with further analytical steps (Penniall, 1966); the sensitivity was low and often required concentration of the color (Munro and Fleck, 1966).

On the other hand, the latter method is characterized by using nitric acid for a rapid destruction of the organic material. Nitric acid is completely volatilized on heating, and leaves no residual acidity. The addition of calcium to the digestion mixture prevents the loss of phosphorus by volatilization or its rearrangement to a less reactive form by maintaining it in the phosphate state. The stabilization of the final heteropoly blue complex is achieved by the addition of the arsenite-citrate reagent which eliminates the escess molybdate by complexing it (Baginski, Fao and Zak, 1967b).

Unless extreme care were taken during the digestion step, the reproducibility was questioned for both methods. The authors of the latter method claimed that the results of heating time did not cause any variation of phosphate or its rearrangement to an unreactive form.

The values of the lipid phosphorus determined by both methods were comparable and agreed with data reported by others (Draml, 1966; Gjone and Orning, 1966; Shih, 1962).

The Baginski, Fao and Zak (1967b) method was adopted because of the advantages of rapidity, simplicity, and sensitivity. The procedure is described in detail in the Appendix, C-4.

Determination of triglycerides

Glycerides were usually calculated from values for total lipids, lipid phosphorus, and cholesterol. The difference between total lipids and the sum of phospholipids and total cholesterol was regarded as representing neutral lipids (Bragdon, 1951). In such a procedure, the errors of separate determination were

compounded in the final estimation of triglyceride concentration. In recent years both chromatographic and direct procedures have been developed.

A batch elution technqiue using florisil (magnesium silicate) was described by Michaels (1962) for isolation of plasma glycerides. A more specific method uses thin-layer chromatography (TLC) for selective isolation of triglycerides and infrared photometry for quantitation (Krell and Hashin, 1963). Quantitative determination of plasma triglycerides by spot size measurement after TLC has been described by Schlierf and Wood (1965).

The most specific component of glycerides is glycerol. Therefore several methods for quantitation of glycerides are based on the determination of the amount of glycerol which is liberated by alkaline hydrolysis. Pertinent methods were published by Van Handel and Zilversmit (1957), Carlson and Wadstrom (1959), Randrup (1960), and Blankenhorn, Rouser and Wiemer (1961). In these procedures glycerol is oxidized with periodate to formaldehyde which is measured colorimetrically after reaction with chromotropic acid and phenylhydrazine. The chromotropic acid procedure was modified by Lofland (1964) for semi-automated determination of serum glycerides. The liberated glycerol can also be condensed with o-aminophenol which in turn is oxidized to yield 8-hydroxyquinoline which can be measured fluorimetrically (Mendelsohn and Antonis, 1961).

Since the elimination of phosphoslipids is necessary in these methods a more specific procedure was developed by Eggstein and Kreutz (1966) which is based on enzymatic determination of free glycerol (Kreutz, 1962). After alkaline hydrolysis without extraction of lipids or isolation of glycerides, glycerol

liberated only from glycerides is assayed with glycerokinase. This procedure is applicable to glyceride determination in serum, homogenates, incubation mixtures etc. A similar method using the enzymatic estimation of glycerol according to Wieland (1957) was described by Spinella and Mager (1966).

Three most recently published methods were selected to be investigated. A colorimetric method was described by Fletcher (1968), in which the glycerol released by saponification of the serum glycerides was oxidized to formaldehyde which reacted with acetyl acetone to form a yellow dihydrolutidine derivative absorbing at 405 mμ. It is interesting to note, that in the method published by Sardesai and Manning (1968) a similar procedure was carried out and the formaldehyde was also estimated colorimetrically by the Hantzsch reaction as described by Nash (1953). The absorbance was determined at 412 mμ instead of 405 mμ. According to the procedure of Galletti (1967), the steps included extraction of nonphospholipidic serum lipid components by isopropyl ether in the presence of silicic acid (Mendelsohn and Antonis, 1961), saponification of glycerides, oxidation of the liberated glycerol with periodic acid and determination of the formaldehyde formed. The color reaction used is a modification of the Schryver's reaction, different from the other two, of formaldehyde with phenylhydrazine and potassium ferricyanide giving a red color, 1, 5-diphenylformazen (Matsui, Okada and Ishidate, 1965; Sawicki and Hauser, 1960).

Comparison of the precision and accuracy of the three methods by performing of recovery and reproducible tests indicated no significant differences. The values of serum triglycerides comparably agreed with the data reported by others (Buckley, Cutler and Little, 1966; Carlson, 1960; Carlson and Wadstrom,

material to be processed. These aspects were discussed extensively by Wren (1960). With careful observation of these rules chromatography is a powerful tool, as was shown by Wren and Mitchell (1958) who were able to separate plasma lipids into 20 different fractions.

Chromatographic separations can also be performed with thin-layers of silica gel and other adsorbents on glass plates (thin-layer chromatography, TLC). This method was standardized by Stahl (1958). Weicker (1959) first applied this technique in the lipid field by separating serum lipids. A comprehensive survey of TLC of lipids was given by Mangold (1962, 1964). According to Wagener (1967) TLC is superior to column chromatography with regard to speed of separation and quality of resolution. Furthermore, depending on the thickness of the adsorbent layer, analytical and preparative work is possible.

Thin-layers for separation of lipids are prepared by spreading a suspension of silica gel (and calcium sulfate) in water on carefully cleaned glass plates. The plates are dried at room temperature and activated at 100-110 C immediately before use and the lipids dissolved in chloroform, benzene or hexane are applied as spots or streaks. For development the plates are brought into glass chambers containing the liquid phase. When the solvent front has reached a certain height the plates are removed and dried. For visualization of separated lipids the chromatoplates may be sprayed with suitable dyes or reagents. Lipids may also be located under ultraviolet light without any pretreatment (Wagener, 1965), or detected with iodine vapor.

Solvents may be used singly or as mixtures for development of the thin-layer plates. Repeated development with the same or other solvents is also possible (Weicker, 1959). In this way a complete separation of neutral lipids (Freeman and West, 1966) and also of neutral lipids plus phospholipids (Vogel, Doizaki, and Zieve, 1962) on a single chromatoplate is possible.

Separation of total lipids is achieved by development with mixtures of hexane, petroleum ether, benzene, chloroform, or diethyl ether. The addition of acetic acid is recommended for clear separation of fatty acids. The main lipid classes are thus separated. After visualization of the individual fractions by non-destructive treatment, the small parts of the chromatoplate corresponding to reference areas can be scraped off the plate and the separated compounds eluted with solvents. The eluted material can be processed further.

Two solvent systems of thin-layer chromatographic procedures were investigated. The procedures of Maier and Mangold (1964) and Pie and Giner (1966) were compared. The latter system showed better separation and this system was adopted. The merits of this TLC were the rapidity and reproducibility. The one disadvantage was the larger surface of lipid that was exposed to air. The procedure described in detail is given in Appendix C-6.

Quantitative analysis by TLC was investigated as a means of determining the distribution of lipid classes in total lipid extracts. The least troublesome approach for analysis is spot densitometry of the plate (Scott, 1969). Purdy and Trueter (1962a; 1962b) reported that the amount of substance could be determined from the size of the spot it formed, irrespective of the intensity of the color.

However, many attempts to verify the weight-area relationship for lipid classes were not successful.

Gravimetric measurements of the lipid classes isolated by the TLC also were investigated but were not successful either. The adsorbent contaminating the samples could not be removed sufficiently for accurate weighings of the micro amounts of lipids available.

When quantification by TLC and gravimetric analyses proved to be unsatisfactory, the distribution of lipid classes was quantitated by direct chemical analyses of the total lipid extract for cholesterol, lipid phosphorus and triglycerides. In addition to the chemical analyses of the total lipid extracts, a thin-layer chromatogram of each of the samples was prepared which allowed for some semi-quantitative visual evaluation.

Gas-liquid chromatographic analysis of fatty acids

Gas-liquid chromatography (GLC) is the most available instrumental technique for the analysis of fatty acids. In combination with liquid-solid chromatography and thin-layer chromatography, it has become a very powerful tool both in industry and research. Particularly in the biomedical sciences, GLC is an invaluable tool for studying lipid metabolism by the use of C^{14} and H^{3} labeled compounds.

The first gas chromatographic separation of fatty acids was performed by James and Martin in 1952. The chromatograms they obtained separating free fatty acids cannot now be considered satisfactory; but, technology has improved since then.

Gas-liquid chromatography is now widely used for the separation, identification and estimation of long-chain fatty acids and related substances. The basic principles are well recognized, and methods for qualitative work, involving the separation and identification of individual components of a mixture are well established.

Lipids can be isolated from blood in tissue by the extraction method developed by Folch, Lees, and Sloane-Stanley (1957). This method gives a pure lipid extract, free from water soluble materials such as amino acids, peptides, and carbohydrates. It has been previously discussed in the total lipid extraction section.

The fatty acids are usually converted to methyl esters for GLC work. It would be equally possible to use other esters, but a large body of data based on methyl esters has been accumulated. The fatty acids from the saponification procedure or free fatty acids from any other source can be methylated with different procedures (see Caster, 1969; Hammarstrand, 1966). Direct conversion to fatty acid methyl esters from lipids can be done by use of H_2SO_4-methanol or HCl-methanol (Stoffel, Chu, and Ahrens, 1959) or by use of sodium methoxide (Luddy, Barford and Riemenschneider, 1960; Metcalf, Schmitz, and Pelka, 1966). These methods involve the use of sublimation or silic acid column chromatography to free fatty acid methyl esters from the sterols.

Columns from five to twenty feet long are used with an O. D. of 1/8-inch to 1/4-inch. Column size depends on the amount of sample, complexity of sample, and desired resolution.

Different columns have been used for fatty acid analysis. For the analysis of the methyl esters, both polar and non-polar columns are being used. The most common polar columns are the polyesters like diethylene glycol succinate (DEGS), diethylene glycol adipate (DEGA), and so on. These columns will give a complete separation of the fatty acids including those with double bonds.

The two most common non-polar columns are silicone polymers like SE-30 and hydrocarbon greases such as Apiezon L. The SE-30 will only separate the saturated acids, while the Apiezon L will separate the unsaturated except for those with two and three double bonds (linoleic and linolenic) which appear as one peak.

In order to quantitate a fatty acid mixture, the response and linearity of the detector must be known. The best way to test for adequate qualitative and quantitative performance of the column is to use reference mixtures frequently.

Percentage composition calculations are commonly carried out; for this it is necessary to obtain an area measurement associated with each component. Many different procedures have been proposed for area calculations. Multiplication of peak height by width at half-height is preferred in most of the laboratories (Horning et al., 1964). However, it must be recognized that serious errors may be introduced in measuring the widths of very narrow or very broad peaks.

It has been shown (Ettre and Kabot, 1963) that the relative response of fatty acid methyl esters using the flame detector can be taken as weight concentration. The thermal-conductivity detector does not directly represent molar or weight concentration (Horning et al., 1964). A correction factor should be used.

The methyl esters of fatty acids of cholesterol esters, phospholipids, and triglycerides were prepared by interesterification according to the method of Stoffel, Chu and Ahrens (1959), modified by Smith (1965). The procedures in detail are given in Appendix C-7.

Determination of Urinary Estrogens

Procedures for the determination of estrone, 17β-estradiol and estriol in urine are described in detail in a manuscript previously prepared by the author (1967).

Statement of the Hypotheses

Studies on healthy nonpregnant females suggest that the urinary excretion of estrogens is cyclic and changes during the menstrual cycle. The excretion is least during the first week of the cycle; rises to reach a peak at about the mid-cycle; probably decreases transiently; and then rises to a second peak between the third and fourth week of the cycle. With onset of menstruation the low level appears again.

There is ample evidence of the estrogenic hormonal influence on circulating lipids. Observations in the cyclic changes of the serum lipids in young women correlate well with estrogenic activity during the menstrual cycle (Oliver and Boyd, 1953). Information is limited, however, as to the normal variations in distribution of serum lipid classes and the fatty acid patterns of each individual lipid fraction in relation to the endogenous estrogen levels produced in the body during the menstrual cycle of healthy nonpregnant menstruating women.

In order to obtain more information concerning the above relationships, it is hypothesized that

1. The variation of levels of urinary estrogen and serum lipids of each individual is less than that found among a group of non-pregnant menstruating women who are maintained under usual homeliving conditions.
2. If fatty acid composition of serum lipid fractions is influenced by the menstrual cycle, the variations in the fatty acid patterns of the individuals would be detectable in different phases of the cycle.
3. It will be a reciprocal relation between the levels of serum lipids and urinary estrogens.
4. Interrelationships between levels of urinary estrogens and composition of serum fatty acids in different serum lipid fractions will be evidenced by similar variations in the two bio-indices which will occur at the same phase of the cycle.

RESULTS AND DISCUSSION

Since the days on which the blood and urine specimens were taken corresponded to different stages in each menstrual cycle, and as the cycles varied in length from 23 to 38 days, the following procedure was adopted in an attempt to superimpose the graphs.

1. Four reference points were taken in each menstrual cycle; the beginning and end of menstruation, the day of ovulation as determined by the mid-cycle change in morning waking temperature or the levels of urinary excretion of estrogens, and the beginning of menstruation (in the next cycle). These positions were plotted as 1, 4, 14 and 28 days, respectively.

2. Five additional points were fixed as follows: 6 and 3 days before ovulation, 3 and 6 days after ovulation, and 4 days before menstruation. Thus the values at each point during a cycle were plotted as if the cycle were of 28 days.

3. The arithmetical mean for each component (serum total cholesterol, phospholipids, triglycerides, total lipids and fatty acid compositions, urinary estrone, estradiol, estriol and total estrongens) was obtained from the series of observations for each subject during one complete cycle.

Serum Lipids

The concentrations of serum lipids of the twelve subjects are presented in Table 6 and Appendix A (Tables 21-32).

Table 6. Mean values of serum cholesterol (CH), phospholipids (PL), triglycerides (TG) and total lipids (TL) of the subjects

Subject	TL	CH	PL	TG
		mg / 100 ml		
AM	467 ± 24[a]	116.5 ± 3.4	151.5 ± 9.3	96.9 ± 7.8
CO	438 ± 16	138.3 ± 5.7	165.8 ± 6.1	59.2 ± 8.9
CP	505 ± 16	153.3 ± 2.3	169.0 ± 11.3	101.5 ± 8.3
HL	630 ± 30	208.5 ± 5.1	250.4 ± 10.8	114.5 ± 9.3
JB	491 ±33	163.7 ± 5.2	140.7 ± 7.6	173.1 ± 26.6
JE	463 ± 21	135.0 ± 10.8	110.6 ± 4.0	90.8 ± 7.9
LR	604 ± 24	155.3 ± 4.7	202.4 ± 10.1	96.0 ± 11.6
MA	477 ± 19	133.6 ± 4.1	186.4 ± 9.7	131.8 ± 17.4
MC	464 ± 10	134.1 ± 4.8	185.7 ± 8.6	58.5 ± 3.0
MH	569 ± 11	191.3 ± 11.9	217.4 ± 6.5	91.0 ± 9.7
RM	939 ± 31	289.8 ± 8.2	249.1 ± 14.3	186.4 ± 11.6
SL	487 ± 20	127.7 ± 5.1	120.4 ± 3.4	117.0 ± 15.4
MEAN	545 ± 40	162.3 ± 13.9	164.8 ± 8.6	115.1 ± 10.5

[a]Standard error of mean.

All the values of serum lipids are expressed as mg per 100 ml of serum except the compositions of fatty acids of each lipid fraction which are the percentages of the total fatty acids in each individual fraction.

Total lipids

The overall mean for total lipids was 544 with a standard error of 40. The standard errors among the subjects were large compared to that for each subject (36 to 188 among subjects and 10 to 33 for each subject). The range of individual values was 438 to 939. All the values except one, 939 for subject RM, were within the normal range cited by Albritton (1952).

The subject RM came from a family with a history of high serum lipid levels, hence this hereditary factor should be considered.

Total lipids varied not only from person to person but also from day to day for the individual subjects. Values showed less day-to-day variation for each subject than among the twelve, which would be expected (Cromie, et al., 1963). This finding points up the variations from subject to subject in lipid metabolism. And it also indicates that many individuals tend to maintain a definite pattern of lipid metabolism when other conditions remain fairly constant. (There is need for more subjects to build up reliable means for any population segment.)

Distribution of lipid classes

The distribution of lipid classes of the 12 subjects, Table 6, varied from person to person but certain characteristic patterns were apparent. The distribution was characterized by a greater concentration of cholesterol and phospholipids with lower levels of triglycerides. Overall mean percentages

of phospholipids and triglycerides showed good agreement with values reported in the literature (Table 1). Cholesterol values found in this study could not be compared to those reported by some investigators (Table 1), since no total cholesterol values have been reported that were calculated on the basis as percentages of the total lipid values, and not in actual grams. The overall mean values (and ranges) of each lipid fraction were as follows: cholesterol, 162 (117-290); phospholipids, 166 (64-256); triglycerides, 115 (48-289). These findings were quite similar, except for one value, 290 (subject RM), to that reported by others (Eggstein and Kreutz, 1966; Fletcher, 1968; Galletti, 1967; Lewis et al., 1957; Martinek, 1965; Masoro, 1968; Sardesai and Manning, 1968; and Wagener, 1967).

The day-to-day variations of lipid classes of each subject and the variations from person to person among the 12 subjects were about the same, as evaluated by a comparison of the standard errors of the means. In Tables 6, and 21-32, it is apparent that phospholipids and cholesterol showed least variation. Triglycerides showed the greatest variation, which would be expected, since triglycerides are the most variable components of the plasma lipids (Anonymous, 1971; Masoro, 1968). Even a normal population under postabsorptive conditions exhibits a wide range of plasma triglyceride levels. The level of plasma phospholipids, which under postabsorptive conditions varies far less than triglycerides (Masoro, 1968). Plasma cholesterol, like other lipid fractions, varies widely from individual to individual and much study has been given to the reasons for such differences.

Heredity is one factor that has been cited (Friedman, 1968); this suggested that the blood cholesterol is set by the individual's genetics. This

indicates that subject RM probably had inherited the trait of high cholesterol values (289).

Age is another factor which may affect blood lipids. That cholesterol is the chief component concerned has been indicated by the work of Keys and his co-workers (1949). However, there is some difference of opinion as to whether there is any effect of age on blood cholesterol. Subject HL, 40 years old, had higher cholesterol (209) as well as total lipid (631) values compared to that (128 to 167, cholesterol; 438 to 569, total lipid) of the younger ones (18 to 23 years of age) even though her serum lipid values were within normal ranges. No definite conclusion of the age to serum lipid concentration relationship could be drawn because no data for her (HL) early years were available for comparison.

Fatty acid composition of lipid classes

The percentages of fatty acids combined in cholesterol esters, phospholipids and triglycerides for twelve subjects are presented in Tables 7, 8, and 9, respectively. The mean values and the standard errors of the means for all classes are presented in Table 10. The values are expressed as percentages of the total fatty acids which were resolved by gas-liquid chromatography. Some minor components are not tabulated. The commonly accepted abbreviations of fatty acids were according to number of carbon atoms and double bonds. The fatty acids found in serum and recorded in the tables are as follows: Myristic, 14:0, plamitic, 16:0, palmitoleic, 16:1 stearic, 18:0; oleic, 18:1; linoleic, 18:2; linolenic, 18:3. Some unidentified fatty acids were listed in the tables as "?." The designation of $< 14:0$ and $> 18:3$ referred to the total of one or more acids

Table 7. Mean percentage of major fatty acids of cholesterol esters of the subjects on day of menstrual cycle

Fatty acid	Day of cycle 1	4	8	11	14	17	20	24	28	Mean
	Percent fatty acid									
16:0	10.7±1.6[a]	10.2±3.8	11.3±3.8	10.1±2.3	14.2±4.2	12.1±2.7	12.2±1.9	11.5±2.4	10.5±3.0	11.4± .4
18:0	6.3±1.9	4.2±1.3	6.9±2.9	4.3± .9	6.0±1.1	4.9±1.1	5.6±1.3	3.4± .6	5.6±1.6	5.2± .4
18:1	19.0±2.2	10.2±1.7	21.0±3.8	36.8±9.0	30.1±5.2	30.8±6.3	22.0±2.2	21.2±2.0	27.6±3.6	24.4±1.3
18:2	47.4±8.3	55.4±5.4	49.9±7.6	48.9±7.5	45.2±7.5	49.0±8.6	55.9±7.1	53.0±7.4	56.8±9.7	51.3±1.4

[a]Standard error of mean.

Table 8. Mean percentage of major fatty acids of phospholipids of the subjects on day of menstrual cycle

Fatty acid	Day of cycle									
	1	4	8	11	14	17	20	24	28	Mean
	Percent fatty acid									
16:0	25.1±2.7[a]	29.1±2.9	28.8±6.2	24.6±3.7	23.9±2.2	22.8±2.7	34.1±1.7	34.4±1.9	29.7±3.5	28.1±1.4
18:0	12.7±2.5	12.2±1.3	13.7±3.8	7.1±1.1	12.1±1.9	10.6±1.8	13.1±3.0	13.1±2.0	15.0±3.3	12.2± .8
18:1	14.5±5.1	9.7±1.5	23.9±2.3	5.9± .8	18.2±6.1	14.7±8.5	9.3±2.1	18.9±6.2	21.8±8.2	15.2±2.0
18:2	18.0±7.4	23.0±6.3	18.2±5.2	27.4±10.4	19.2±7.0	22.4±11.3	21.2±7.5	15.5±7.2	21.3±5.2	21.5±2.7

[a]Standard error of mean.

Table 9. Mean percentage of major fatty acids of triglycerides of the subjects on day of menstrual cycle

Fatty acid	Day of cycle									
	1	4	8	11	14	17	20	24	28	Mean
	Percent fatty acid									
16:0	27.0±2.9[a]	24.8±4.0	21.8±3.8	19.0±2.8	25.2±1.7	27.6±3.6	33.2±3.4	28.8±4.9	25.0±3.3	25.8± .7
18:0	7.3±1.1	7.1±2.3	8.9±3.1	3.6± .6	6.8± .8	8.2±2.1	8.7±1.6	8.0±2.4	10.2±1.8	7.6± .6
18:1	33.6±6.7	11.4±1.9	25.0±5.8	43.4±3.4	33.0±5.9	24.1±4.6	31.2±4.5	34.6±9.4	32.1±2.8	29.8±3.0
18:2	14.6±2.0	16.3±3.4	13.5±5.1	14.6±2.0	14.6±1.4	13.3±2.8	10.6±2.8	15.3±3.0	13.1±2.5	13.9± .6

[a]Standard error of mean.

Table 10. Mean percentages of fatty acids of three lipid classes of the subjects

Lipid class	< 14:0	14:0	?	16:0	16:1	?	18:0	18:1	18:2	18:3	> 18:3
	Percentage fatty acids										
Cholesterol esters	0-3.5	14±.2[a]	0-4.7	11.4± .4	3.3±.2	0-4.7	5.2±.4	24.4±1.3	51.3±1.4	0-4.9	0-18.9
Phospholipids	0-1.6	1.0±.4	0-1.1	28.1±1.4	1.3±.3	0-1.3	12.2±.8	15.2±2.0	21.5±2.7	--	0-11.8
Triglycerides	0-7.3	1.3±.5	0-1.8	25.8± .7	5.1±.5	0-1.9	7.6±.6	33.0±2.6	13.9± .6	0-2.5	0-13.8

[a]Standard error of mean

appearing before myristic acid or after linolenic acid which were not identified or were present in too small an amount to isolate adequately for accurate analysis.

The fatty acids which were present in the highest concentrations in serum were palmitic, stearic, oleic and linoleic, the patterns similar to data reported in other studies (Table 2). In cholesterol esters linoleic was present in highest concentration with palmitic and oleic second. The mean percentages of the major fatty acids in cholesterol esters were palmitic 11; stearic, 5; oleic, 24; and linoleic, 51. These values agree well with those reported by others (Table 2). The amount of linolenic acid for one of the subjects one day was much higher than values usually reported (values up to 11 per cent) although the amount for most of the days was only a trace or none.

The proportions of the different fatty acids in phospholipids showed high values in palmitic, low in stearic and oleic acids. The mean values of the major fatty acids in phospholipids were palmitic 28, stearic 10, oleic 15, linoleic 22. The data were similar to those reported by other investigators (Table 2).

In triglycerides, oleic was present in highest concentration followed by palmitic and linoleic with lower amounts of stearic. The mean values of the major fatty acids were: palmitic 26; stearic 8; oleic 33; linoleic 14. These values were comparable with other studies (Table 2). In general, the individual day to day variations of values of the fatty acid combinations of all lipid fractions were equal to or greater than the person-to-person variations found among the twelve subjects, as evaluated by a comparison of the standard errors of the

means. There were, in addition, a few samples of lipid classes which contain distinctly higher amounts of certain fatty acids. For example, linolenic acid in triglycerides for subject RM was 29 per cent and the unidentified acid in phospholipids, with retention time longer than linolenic acid, for subject LR, 12 per cent.

Effect of menstrual cycle

The results of serum cholesterol, phospholipids, triglycerides and total lipids on different stages of the menstrual cycle are presented in Tables 11 to 14. The graphic presentation of data of serum lipids from Tables 11, 12, 13, and 14 are Figures 1 and 2. When the serum lipids of the twelve subjects were plotted, it was observed that the concentrations of total lipid and each lipid fraction underwent regular cyclical changes. The values dropped at the mid-cycle point and also fell to a low value immediately before the onset of menstruation. The findings confirmed the study of Oliver and Boyd (1953) who indicated that in women, serum total cholesterol and phospholipids varied with the menstrual cycle. During the follicular phase of the menstrual cycle up to the point of ovulation, cholesterol and phospholipids increased, but fell abruptly at ovulation. These levels rose again slowly through the luteal phase of the menstrual cycle and again fell abruptly just before menstruation. In this study the cyclical change of serum lipid levels seemed not so pronounced as that indicated by Oliver and Boyd (1953). The concentrations of all serum lipid fractions including total lipids were elevated to reach a very high point in the follicular phase then decreased to a low level before ovulation and kept at this low level until the luteal phase. Then it slowly

Table 11. Serum cholesterol values for individual subjects on day of menstrual cycle

	Day of cycle								
Subject	1	4	8	11	14	17	20	24	28
	mg/100 ml								
AM	--	113.7	--	112.0	135.1	98.3	113.7	110.3	109.4
CO	133.5	--	--	139.6	--	--	146.7	136.0	--
CP	164.3	--	157.0	158.9	--	139.2	150.7	--	--
HL	233.1	--	--	216.3	175.4	--	221.6	197.4	217.4
JB	157.4	--	--	--	155.6	192.6	--	176.9	157.4
JE	176.0	--	--	172.8	63.2	121.6	--	140.0	136.8
LR	171.6	--	--	166.2	145.8	133.4	178.7	160.9	141.4
MA	128.7	--	--	125.5	156.1	135.1	141.5	--	111.4
MC	131.8	167.1	128.3	--	110.9	130.1	--	155.4	128.3
MH	141.6	--	172.8	--	206.4	--	217.6	223.2	164.0
RM	281.6	262.8	--	--	269.3	295.8	268.3	287.2	--
SL	139.4	--	156.5	--	115.4	139.4	113.7	--	130.8
Mean	169.0±14.4[a]		153.9±9.3		153.3±7.8		172.5±17.5		144.1±11.0
		187.6±53.7		155.9±13.1		153.9±19.6		176.4±17.8	

Overall Mean: 162.9±4.7

[a]Standard error of mean.

Table 12. Serum phospholipid values for individual subjects on day of menstrual cycle

Subject	Day of cycle 1	4	8	11	14	17	20	24	28
					mg/100 ml				
AM	--	147.1	--	117.5	129.0	145.9	142.3	153.5	163.6
CO	170.0	--	--	179.5	--	--	189.7	153.6	--
CP	206.2	--	167.2	116.1	--	221.4	155.3		
HL	262.6	--	--	261.0	220.5	--	320.4	236.0	255.7
JB	122.9	--	--	--	111.2	184.0	--	145.6	139.1
JE	106.1	--	--	95.9	108.4	107.7	--	104.0	101.2
LR	179.3	--	--	235.4	257.6	204.4	201.4	179.1	204.4
MA	179.2	--	--	179.8	208.6	201.5	139.0	--	140.3
MC	195.0	160.6	142.6	--	175.6	201.0	--	217.6	222.0
MH	183.0	--	186.1	--	170.7	--	160.8	202.0	194.3
RM	268.8	330.0	--	--	218.4	235.6	212.1	266.3	--
SL	139.9	--	125.2	--	129.9	108.9	129.2	--	106.8
Mean	186.3±5.7[a]		164.6±21.4		176.5±16.8		187.0±19.5		173.8±18.9
		212.6±58.8		169.3±23.9		178.9±15.7		188.7±18.1	

Overall Mean: 182.0±4.7

[a]Standard error of mean.

Table 13. Serum triglyceride values for individual subjects on day of menstrual cycle

Subject	Day of cycle 1	4	8	11	14	17	20	24	28
					mg/100 ml				
AM	--	131.5	--	98.4	82.8	112.6	70.2	141.0	78.6
CO	66.6	--	--	40.5	--	--	60.0	21.1	--
CP	110.5	--	81.9	145.0	--	109.5	86.5	--	--
HL	113.9	--	--	98.3	88.2	--	115.9	115.9	106.5
JB	116.4	--	--	--	104.7	145.2	--	72.9	--
JE	109.5	--	--	120.6	80.7	114.6	--	40.8	103.8
LR	58.7	--	--	29.8	141.1	113.3	117.9	93.6	56.9
MA	117.8	--	--	134.1	82.1	--	134.9	--	81.3
MC	55.4	53.7	59.0	--	76.2	45.7	--	58.6	62.5
MH	68.4	--	138.6	--	77.8	--	69.2	91.3	111.3
RM	147.9	221.4	--	--	166.8	156.0	187.5	145.8	--
SL	78.4	--	87.4	--	91.2	65.8	200.9	--	159.6
Mean	94.9±9.2[a]		91.7±22.4		99.2±9.7		115.9±17.1		95.1±11.7
		135.5±48.5		95.2±16.6		107.8±13.0		94.1±16.8	
Overall mean:	103.3±4.3								

[a]Standard error of mean.

Table 14. Serum total lipid values for individual subjects on day of menstrual cycle

Subject	Day of cycle 1	4	8	11	14	17	20	24	28
	mg / 100 ml								
AM	--	518	--	292	425	420	556	527	474
CO	474	--	--	527	--	--	469	382	--
CP	547	--	493	428	--	586	508		
HL	395	--	--	714	612	--	632	569	678
JB	549	--	--	--	345	622	--	583	433
JE	423	--	--	330	488	472	--	500	413
LR	484	--	--	646	722	649	714	488	639
MA	445	--	--	527	537	513	513	--	425
MC	462	457	452	--	423	513	--	474	420
MH	544	--	625	--	552	--	535	576	581
RM	1047	1050	--	--	979	982	819	875	--
SL	490	--	507	--	540	389	522	--	395
Mean	533±40[a]		519±37		562±57		585±38		495±36
		675±188		499±59		572±59		553±45	

Overall mean: 554±19

[a]Standard error of mean.

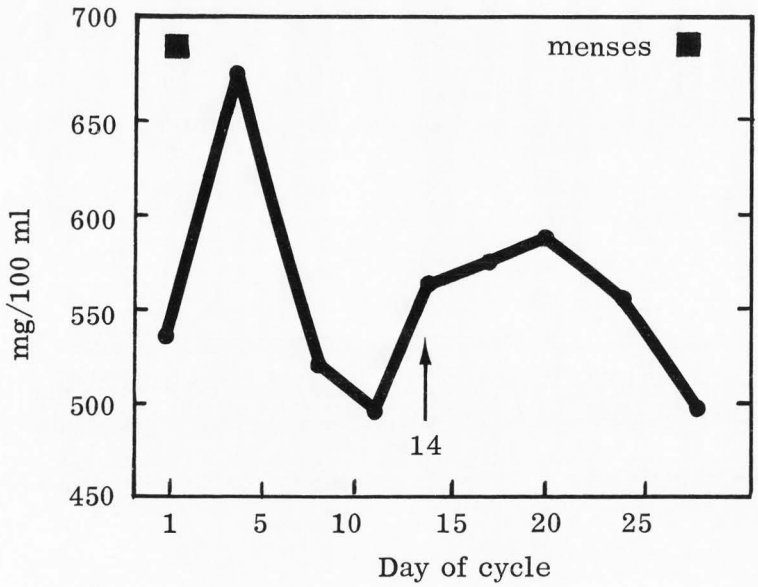

Figure 1. Mean values of serum total lipids of the subjects on day of menstrual cycle.

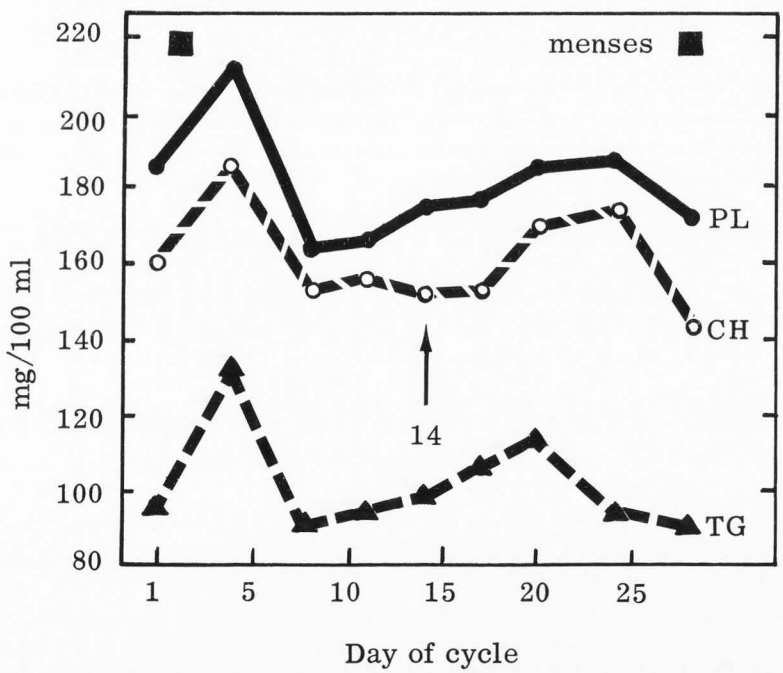

Figure 2. Mean values of serum total cholesterol (CH), phospholipids (PL), and triglycerides (TG) of the subjects on day of menstrual cycle.

increased reaching a second peak but lower than in the follicular phase. At the end of the cycle or immediately before the onset of menstruation, the serum lipid levels fell again to point equal to or even lower than that at the ovulation time, with exception of triglycerides, which showed somewhat higher values than other lipid components.

The observation that the low values for the serum lipids occurred at or about the time of ovulation and just prior to menstruation suggests that these cyclic changes may be directly or indirectly influenced by the female hormones. The secretion of the estrogenic hormones, judged by quantitative measurements of urinary estrogens, is generally believed to be maximal immediately before ovulation, and to reach a secondary peak just before menstruation (Brown, 1960). There is, indeed, some direct experimental evidence to support this view. Administration of estrogens to men and to postmenopausal women results in a decrease in the level of plasma cholesterol, reduction in cholesterol to phospholipid ratios, and a relative increase in the high density α-lipoproteins. There is no doubt about the ability of extraneous estrogen hormones to influence plasma lipid patterns in man (Furman et al., 1958; Moses, 1963; Musa, Seal and Doe, 1965).

It seems reasonable to suppose, therefore, that in women during their reproductive years the serum lipids are regularly depressed as a result of the secretion of the estrogenic hormone at the ovulation of each menstrual cycle. According to the review of Sulimovici and Boyd (1969), there is considerable evidence to indicate that cholesterol is the obligatory precursor of steroid hormones produced by the ovary, adrenal, and placenta. This strongly supports the role of estrogenic hormones on regulating of serum cholesterols. Albers and Riggi

(1966) indicated that the hypocholesterolemic effect is due to enhanced hepatic sterol sequestration.

Vikrot (1964, 1965) showed that there was a progressive drop in plasma lysolecithin concentrations during pregnancy in humans and rats. Eisenberg, Stein and Stein (1967) confirmed this observation in rats and concluded that lowering of lysolecithin concentrations was probably related to uptake and esterification of lysolecithin by the placenta. Portman et al. (1968) showed the same results on rhesus monkeys. The plasma was shown to contain high concentrations of lysolecithinase activity and the placenta was established as the primary source of this enzymatic activity. Lysolecithinase activity was extremely low in the plasma or serum of males, fetuses and infants, and nonpregnant females at all stages of the menstrual cycle. Svanborg and Vikrot (1966), also showed that lysolecithin concentrations were reduced when estrogens were given to ovariectomized humans. This effect is presumably not the result of decreased lysolecithin formation by the LCAT (Lecithin-cholesterol-acyl transferase) reaction, since Aftergood and her co-workers have already shown that transferase activity is enhanced by the estrogenic hormones and by the oral contraceptive enovid (Aftergood and Alfin-Slater, 1967; Aftergood, Hernandez and Alfin-Slater, 1968).

It is known that most of the serum phospholipids are in the form of lecithin with smaller but significant amounts in sphingomyelin. All other types of phospholipids are present at low levels. Therefore, pregnancy as well as high concentrations of estrogen, as at ovulation, results in a decrease in the concentrations of serum or plasma cholesterol, phospholipids, and other lipids. There is some

evidence (Stamler, 1963) that certain arteries may be selectively protected from atherosclerosis by pregnancy and the hormones of the premenopausal period. It is possible that the low levels of serum lipids may be involved.

Cholesterol and phospholipids which are the two major lipid components of serum in postabsorptive state, contribute approximately 80 per cent of the total lipid. The variation of total lipid levels may be due entirely to the fluctuation of both cholesterol and phospholipids. It is concluded therefore, that the serum total lipid levels in this study followed the same pattern of both cholesterol and phospholipids.

The variation of fatty acid composition in different lipid fractions were discussed previously. In general, day-to-day variation of values for individual subjects were equal to or greater than the individual-to-individual variations found among the twelve subjects. Differences in the degrees of variation of specific fatty acids in the three lipid classes on day of menstrual cycle are more apparent in a graphic presentation of data for the major fatty acids from Tables 7, 8, and 9, Figures 3 to 5. Of all the fatty acids plotted, the ones with the smallest amount of fatty acids varied the least . This was true for both cholesterol esters and triglycerides; palmitic (16:0) and stearic (18:0) varied the least for cholesterol esters; stearic and linoleic (18:2) the least for triglycerides. Oleic (18:1) showed the most variation for all three classes. It was reported (Smith, 1965) that the fatty acid varied little in phospholipids. Results in this study, on the other hand, were found to be contradictory in that all the major fatty acids varied to a great extent in phospholipids. Linoleic acid in cholesterol, as well as palmitic acid in phospholipids followed the same pattern with relative low

concentration in the beginning gradually increase in the follicular phase then fell at or before the ovulation, and increase again in the luteal phase. This pattern is quite similar to that of serum lipids. It is interesting that oleic acid in cholesterol esters had the same pattern as the two acids discussed above but in an opposite direction. The fact that this acid increased with a decrease of the other two acids and visa versa. The results of this study failed to confirm a previous study (Sang, 1967) which was conducted in this laboratory.

Effect of dietary intake

During the experimental periods, all of the subjects were asked to record exactly the amount of food that they had consumed the day before urine specimens were taken. In Table 15 the daily intake is presented as calculated for calories, protein, carbohydrate, fat, fatty acids (unsaturated and saturated), and cholesterol. In general, the total calorie intakes of the subjects ranked a little lower than the recommended allowance (1905 vs 2100). Proteins, carbohydrates and fats were used in a well balanced proportion to each other from the nutritional view point. The mean percentage of calories consumed by all subjects as protein was 16; fat 43; and carbohydrate, 46. In general, a medium level of fat intake is considered to include approximately 35 per cent of the calories as fat. These subjects were consuming 43 per cent which is designated as a high intake of fat by most nutritionists. The average diet in the United States as calculated from many studies included as per cent of calories, carbohydrate is 40-50, protein 14-15 and fat 40 to 48.

Table 15. Dietary records of the subjects

Subject[a]	Weight	Calories	Carbohydrates	Proteins	Fats	Fatty acids			Cholesterol
						Sat.	Unsat. Oleic	Unsat. Linoleic	
	lb		gm	gm	gm	gm	gm	gm	mg
AM	130	1773	219	74	100	45	35	10	501
CO	129	1295	112	66	53	14	21	5	489
CP	125	2164	246	89	126	47	41	7	546
HL	170	2297	269	86	94	30	39	15	492
JB	113	2153	257	76	110	25	33	14	557
JE	144	2252	277	95	91	36	34	17	561
LR	128	2106	238	90	105	35	39	11	573
MA	128	1359	156	46	68	24	21	4	439
MC	158	2246	257	89	105	48	38	10	522
MH	129	1873	229	70	70	25	26	9	578
SL	127	1435	148	74	78	34	30	7	570
Mean	135	1905	219	78	91	33	32	10	530
Percent of Calories			46	16	43				

[a]Complete data for RM was unobtainable.

On an individual basis some subjects were not as well nourished as the mean nutrient intakes indicated. The highest intake in grams of protein and of fat was twice the lowest value and the individuals for both nutrients were not the same subjects.

Blood lipid levels are affected by diet and under most conditions are extremely uniform in any one individual. Moreover, the composition of fasting bloods of different normal individuals shows quite constant values. This was true for the present study.

Dietary factors, such as total calories, fat, protein, carbohydrate, etc., influence the level of the blood lipids.

The level of the three main lipid components of blood are interrelated. Conditions affecting the level of any one of these components usually cause changes in the other two.

The most important factor which increases the level of blood lipid is intake of fats. A hyperlipemia, characterized mainly by elevations in triglyceride and nonesterified fatty acid fractions is obtained after fat ingestion. Not only the level of neutral fat, but also that of serum phospholipids is increased by the ingestion of fat (Alfin-Slater and Aftergood, 1968). Quite variable results have been reported on the effect of fat ingestion on the level of blood cholesterol (Nestel, 1970). There are also divergent opinions as to whether or not the intake of cholesterol causes a hypercholesterolemia in man (see Connor, Hodges and Bleiler, 1961; Connor, Stone and Hodges, 1964; Erickson et al., 1964). It has been known for some time that cholesterol absorption may be influenced by a raise in the cholesterol ingestion. This has recently been confirmed by isotope and

sterol balance techniques (Grundy and Ahrens, 1969; Grundy, Ahrens and Davignon, 1959). There is evidence that unsaturated fat has a cholesterol-lowering effect (Connor et al., 1969; Grundy and Ahrens, 1966; Hellman et al., 1957; Moore et al., 1968). The amount and kind of dietary carbohydrate apparently can influence serum cholesterol and lipid metabolism in general (Anonymous, 1971; Herman, Zakim and Stifel, 1970; Hodges and Krehl, 1965).

According to the brief review cited above dietary intake is the most important factor affecting the serum lipid levels. However, in the present study emphasis must be placed on the magnitude of biologic differences. Widdowson (1962) has pointed out that physiologic characteristics, inherent in each one of us, influence nutritional conditions. In normal persons, intake, absorption and utilization of nutrients vary within a wide range, and therefore, inter-individual differences have also been found in the human serum lipid levels (Keys, Anderson and Grande, 1956b; Widdowson, 1962). Besides, physical activity may play as important a role as diet in the regulation of serum lipids (Campbell, 1965; Gsell and Mayer, 1962). From a short time mean food intake and without knowing other environmental and hereditary factors there is no way to evaluate the differences of concentrations in serum lipids of different individuals. And therefore no attempt has been made to draw any conclusion.

Urinary Estrogens

The concentrations of the excretion of estrogens in the urine are presented in Tables 16 to 20, and 33 to 44.

All the values of urinary estrogens are expressed as μg per 24 hours.

Table 16. Mean values of urinary estrone (E_1) 17β -estradiol (E_2), estriol (E_3) and total estrogen (E_t) of the subjects

Subject	E_1	E_2	E_3	E_t
		μg / 24 hour		
AM	13.7±1.1[a]	8.0±0.7	21.7±2.4	43.4±3.9
CO	5.5±0.5	3.0±0.5	13.9±1.2	22.4±2.0
CP	9.2±0.6	6.1±0.7	17.4±1.5	32.7±2.6
HL	9.9±1.2	4.0±0.6	15.1±2.1	29.0±3.7
JB	5.4±0.6	1.0±0.4	8.3±1.2	14.8±2.1
JE	12.6±1.4	7.1±0.6	20.1±1.8	39.8±3.7
LR	9.3±0.8	3.9±0.6	16.8±2.4	29.9±3.5
MA	6.7±0.6	3.8±0.5	15.5±2.9	25.6±3.3
MC	9.6±1.1	3.4±0.4	15.4±1.9	28.4±3.4
MH	9.2±0.8	4.9±0.5	17.7±2.0	32.8±2.8
RM	7.8±0.5	4.0±0.5	14.2±0.9	25.9±1.4
SL	5.3±0.6	8.5±0.8	20.9±2.5	34.7±3.6
Mean	8.7±0.8	4.8±0.6	16.4±1.0	29.9±2.2

[a]Standard error of mean.

Table 17. Urinary estrone values for individual subjects on day of menstrual cycle

Subject	Day of cycle 1	4	8	11	14	17	20	24	28
				μg/24 hour					
AM	-	7.8	-	14.0	20.5	-	11.9	16.4	6.8
CO	3.8	6.2	-	7.4	-	3.5	4.5	10.1	1.9
CP	4.5	6.4	10.1	-	-	7.6	10.6	-	2.5
HL	4.0	5.1	8.0	-	20.1	-	8.9	-	7.3
JB	1.8	-	2.8	5.0	10.6	5.2	-	8.5	3.1
JE	4.1	4.8	12.0	13.1	21.0	11.0	-	18.1	6.7
LR	4.1	6.2	-	8.9	16.4	9.4	-	10.9	7.2
MA	4.3	3.8	-	7.1	11.8	6.1	5.9	-	6.2
MC	4.2	4.5	6.2	8.1	20.2	9.5	-	10.2	7.2
MH	4.5	-	10.6	-	13.4	-	10.2	9.4	5.2
RM	-	-	7.1	5.8	-	6.8	-	8.8	-
SL	N[a]	5.0	3.6	6.7	9.4	2.3	-	-	-
Mean	3.5±.5[b]	5.5±.4	7.6±1.2	8.5±1.0	15.9±1.6	6.8±1.0	8.7±1.2	11.6±1.3	5.4±.7

Overall mean: 8.2±1.2

[a]Not detectable.
[b]Standard error of mean.

Table 18. Urinary 17β-estradiol values for individual subjects on day of menstrual cycle

Subject	Day of cycle 1	4	8	11	14	17	20	24	28
	μg/24 hour								
AM	-	6.0	-	7.5	14.0	-	6.2	9.5	4.3
CO	N[a]	0.8	-	3.3	-	1.8	2.5	6.2	2.1
CP	3.2	4.5	6.7	-	-	0.8	10.1	-	2.5
HL	1.8	2.1	4.5	-	8.8	-	3.0	-	2.5
JB	N	-	N	0.7	6.0	0.2	-	1.8	N
JE	3.2	4.0	7.5	8.5	11.3	7.3	-	7.9	4.8
LH	N	4.1	-	6.7	7.3	3.6	-	6.6	1.8
MA	2.1	N	-	4.8	6.2	2.3	2.1	-	3.7
MC	-	1.8	2.5	3.8	8.0	3.3	-	2.8	2.4
MH	3.3	-	5.9	-	5.2	-	5.2	4.9	2.5
RM	-	-	2.5	3.7	-	3.4	-	5.3	-
SL	4.1	6.2	7.6	10.9	16.4	3.6	-	-	-
Mean	2.0±.5[b]	3.3±.7	2.8±1.0	5.5±1.0	9.2±1.3	3.5±.7	4.3±1.2	5.6±.9	2.7±.4

Overall mean: 4.4±.7

[a]Not detectable.
[b]Standard error of mean.

Table 19. Urinary estriol values for individual subjects on day of menstrual cycle

Subject	Day of cycle 1	4	8	11	14	17	20	24	28
				μg/24 hour					
AM	-	12.1	-	27.0	36.3	-	12.1	22.7	10.1
CO	10.2	9.1	-	15.6	-	9.1	8.1	24.0	9.3
CP	7.9	12.5	22.8	-	-	16.9	31.1	-	11.2
HL	6.5	6.5	10.8	-	30.8	-	15.7	-	14.0
JB	2.5	-	2.0	2.2	16.8	8.0	-	13.1	4.5
JE	12.0	10.9	16.7	19.3	30.7	17.3	-	24.8	13.4
LR	6.8	3.2	-	17.0	34.9	15.2	-	31.1	7.9
MA	6.0	4.1	-	9.8	34.6	19.8	14.7	-	8.5
MC	-	5.3	8.1	10.7	29.5	17.0	-	17.8	12.8
MH	7.9	-	11.2	-	31.1	-	18.5	29.4	9.2
RM	-	-	15.3	12.0	-	9.1	-	14.7	-
SL	6.8	14.5	27.8	22.8	40.4	11.2	-	-	-
Mean	7.4±.9[a]	8.7±1.4	14.3±2.9	15.2±2.5	31.7±2.2	13.7±1.5	16.7±3.2	22.2±2.3	10.1±9

Overall mean: 15.6 ±2.5

[a]Standard error of mean.

Table 20. Urinary total estrogen values for individual subjects on day of menstrual cycle

Subject	Day of cycle 1	4	8	11	14	17	20	24	28
	μg /24 hour								
AM	-	25.9	-	48.5	70.8	-	30.2	48.6	21.2
CO	14.0	16.1	-	26.3	-	14.4	15.1	40.3	13.4
CP	15.6	23.4	39.6	-	-	30.4	51.8	-	20.5
HL	12.3	13.4	23.3	-	59.7	-	27.6	-	23.8
JB	4.3	-	4.8	7.9	33.4	13.4	-	23.4	7.6
JE	19.3	19.7	36.2	40.9	63.0	35.6	-	50.8	24.9
LR	10.9	13.5	-	32.6	58.6	28.2	-	48.6	16.9
MA	12.4	7.9	-	21.7	52.6	28.2	22.7	-	18.4
MC	-	11.6	16.8	22.6	57.7	29.8	-	31.8	22.4
MH	15.7	-	27.7	-	49.7	-	33.9	43.7	16.9
RM	-	-	24.9	21.5	-	19.3	-	28.8	-
SL	10.9	25.7	39.0	40.4	66.2	17,1	-	-	-
Mean	12.8±1.4[a]	17.5±2.2	26.5±4.2	29.2±4.2	56.9±3.6	24.0±2.7	30.2±5.1	39.4±3.7	18.6±1.7

Overall mean: 28.3±4.4

[a]Standard error of mean.

Individual estrogen

The relative amounts of the three estrogens excreted in the urine varied considerably from subject to subject; usually estriol was greater than estrone, and 17β-estradiol (for convenience "estradiol" will refer to 17β-estradiol) was the least of the three. The mean values (and ranges) of estrone (E_1), estradiol (E_2) and estriol (E_3) were 8.7 (0 to 21), 4.8 (0 to 16), and 16.4 (2 to 43), respectively. These results were within the acceptable range for normal young women given by other studies (Table 4).

Total estrogen

The amount of total estrogen is expressed as the sum of the three estrogens. Brown (1960) has indicated that the sum of these three estrogens correlated more closely with the clinical signs of estrogenic activity than the amount of any individual estrogen. The overall mean of total estrogen for the entire study was 29.9. As expected, both the person to person and day to day variations were high. The values of the twelve subjects ranged from 4.3 to 73.8. These results were within the ranges of those reported by others (Table 4).

Effect of menstrual cycle

A characteristic pattern of estrogen excretion was found during the menstrual cycle. This pattern is illustrated in Figure 6 (from Tables 17 to 20), which shows the composite results of the excretion of estrone, estradiol and estriol obtained from the twelve women throughout their menstrual cycles. The amounts of the three estrogens excreted rose and fell together. They were lowest during the first week of a 28-day cycle, and then elevated to a peak, which occurred on or

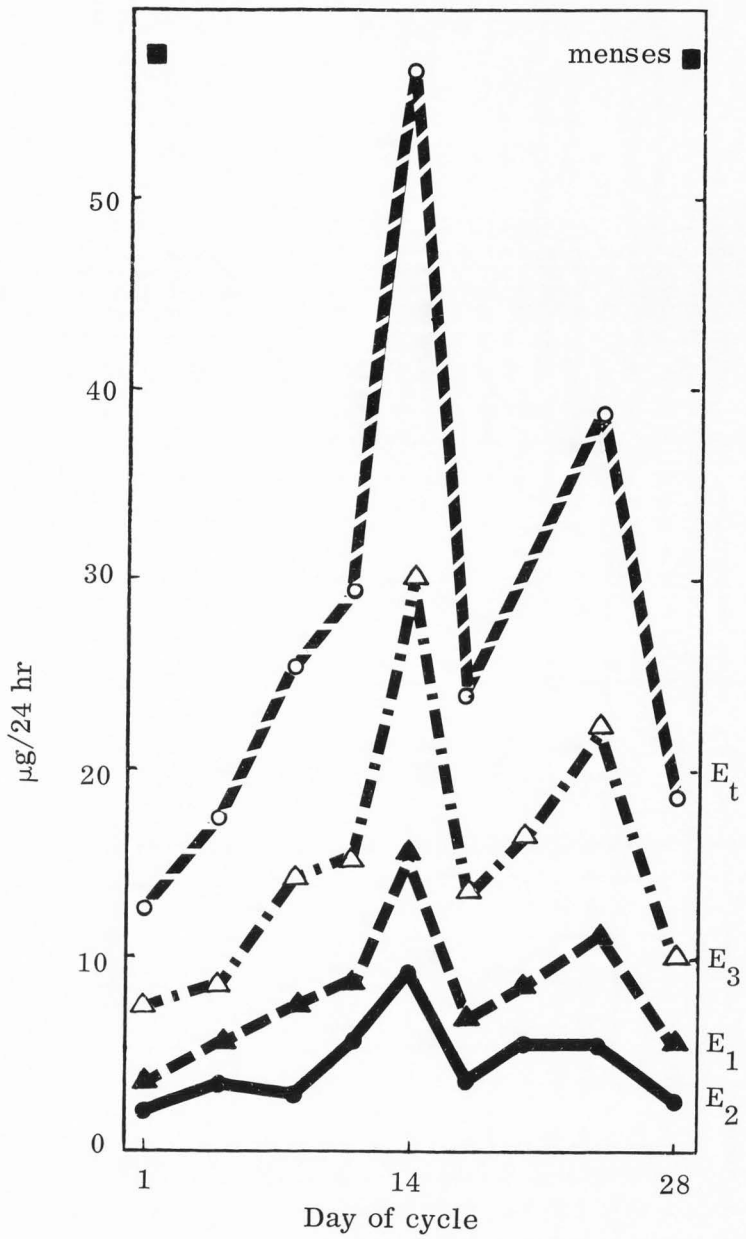

Figure 6. Mean excretion values of estrone (E_1), 17β -estradiol (E_2), estriol (E_3) and total estrogen (E_t) of the subjects on day of menstrual cycle.

about the 14th day of the cycle. The estrogen excretion then fell and rose again to a second peak which was usually lower than the first one, and in some individuals was not well defined. During the last few days of the cycle, the estrogen excretion dropped and menstruation occurred at this time. The mean values of estrone, estradiol and estriol for the first week of the cycle of the twelve women were 4.5, 2.6 and 8.0; 15.9,9.2 and 31.7 for the mid-cycle; 10.2, 6.3 and 19.5 for the time between the third and fourth week; and 5.4, 2.7 and 10.1 for the time immediately before the onset of menstruation. These results were similar to those reported by Brown (1955) Loraine and Bell (1963) and Lee (1965; 1967).

According to Brown (1960) a reasonable explanation for this urinary pattern, based on ovarian function, is as follows: the amounts of estradiol and estrone that was secreted increased as the Graafian follicle increased in size and reached a peak just before rupture at ovulation; rupture causes a temporary arrest in estrogen production and output falls; growth and regression of the corpus luteum leads to the second increase and decrease in estrogen excretion. He also stated that estradiol and estrone are metabolized and eliminated rapidly in the urine as their conjugates and slightly less rapidly as estriol; fluctuations in urinary estradiol and estrone should be closely related in time to the corresponding fluctuations in estrogen production by the ovaries. This assumption supported the fact that the rise and fall of estriol levels often tended to lag slightly behind the increase and decrease of estrone and estradiol levels observed in the present study.

A characteristic ovulatory peak was invariably observed for nine subjects out of twelve. Whenever ovulation occurred, it was indicated by basal temperature

records as well as the rise in urinary estrogen excretion itself. The period of time between the onset of the previous menstrual bleeding and the ovulatory peak varied from 11 to 24 days, and between the peak and the onset of the next menstrual bleeding, from 11 to 16 days. The rise of basal temperature did not always coincide with the estradiol-estrone peak as it sometimes occurred a few days after the estrogen peak. No ovulatory peak was observed in three individuals, therefore attempts timed to coincide with the rise in basal temperature were unsuccessful.

The true significance of changes in excretion values for women can only be observed when day of menstrual cycle is considered.

The mean total estrogen excreted by the twelve subjects from the beginning of the onset of menstrual bleeding to the approximate time of ovulation showed an increase of 44.1μg (Table 16) or 77 per cent. A second and lower peak occurred during the luteal phase of the second half of the cycle, which was 63 per cent (22 μg) above the value at the start of the cycle. This was a decrease of 14 per cent from the first peak. Total estrogen values continued to decrease during the last few days of the cycle. Because of the large variation among subjects, it would be highly desirable to obtain daily estrogen data on more subjects to observe the significance of the effect of the menstrual cycle on this metabolite.

A difficulty encountered in this study was the unpredictable variation of cycle length of some of the subjects, even those who had a very regular length of cycle; therefore, no specimens were collected during certain specified dates of the cycle. It would also be desirable that a longer study be undertaken for example, samples for two or three complete cycles should be collected.

Relationship of Serum Lipids and Urinary Estrogens

A comparison of the patterns shown for the two biochemical indices, the excretion of estrogens and the levels of serum lipids are presented in Figures 7 and 8. Both urinary estrogens and serum lipids were found to have cyclic changes during the menstrual cycle. A reciprocal relationship between the changes was established. The rise in excretion of estrogens produced a decrease in serum lipid components including total lipid levels with the exception of triglycerides which showed slightly different variations. However, the peaks for the two metabolites did not occur on the same day of the cycle. The effect of estrogenic hormones on serum lipid metabolism has been previously discussed. According to Boyd (1963) the endogenous estrogenic hormones appear to influence cholesterol metabolism in at least two ways. There is an effect on the biosynthetic mechanism and also an influence on the rate of degradation or excretion. This was evidence that the point at which estrogen hormones influence cholesterol metabolism lies between acetate and mevalonate (Bucher, Overath and Lynen, 1960). It also suggested that the hormone may affect cholesterol metabolism through an enzyme system.

The effect of estrogen on the composition of fatty acids was found similar to the serum lipids pattern of cholesterol linoleate, and palmitate of phospholipids with an opposite pattern of cholesterol oleate and palmitate. Boyd (1963b) reported that there is a drop in cholesterol linoleate and a rise in cholesterol palmitate in the female human and rat ovariectomy. This finding could be supported, at least in part, by data in the present study.

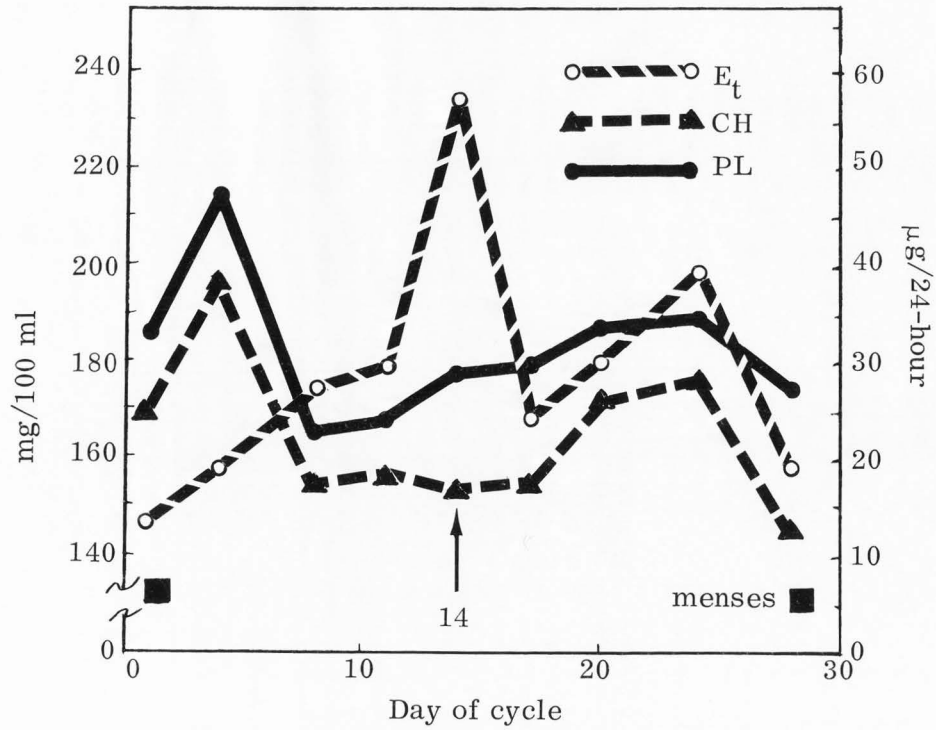

Figure 7. Serum cholesterol and phospholipids and the excretion of urinary total estrogens on day of menstrual cycle of the subjects.

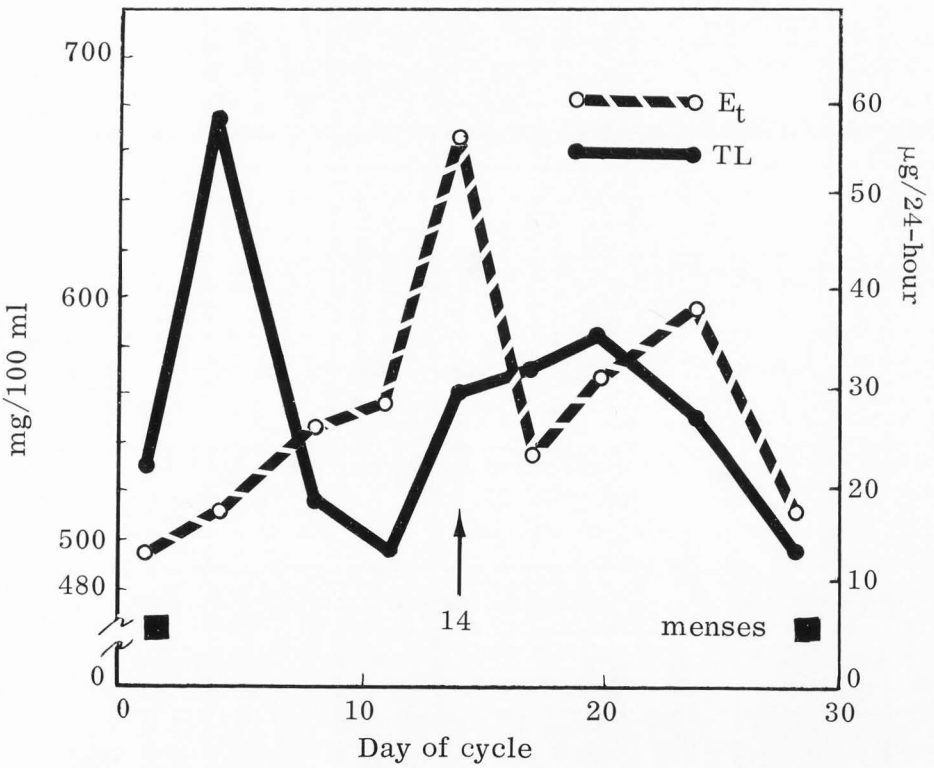

Figure 8. Serum total lipids and the excretion of urinary total estrogens on day of menstrual cycle of the subjects.

In general, endogenous estrogenic (hormonal) effects on lipid metabolism are quantitatively of a lower order of magnitude than other physiological effects. This means that one must view hormone studies in perspective and not over-emphasize their importance in this context. The estrogenic control of lipid metabolism should be looked upon as a fine regulation of complex processes superimposed upon more important controlling mechanisms.

CONCLUSIONS

The results herein reported are based upon the following assumptions: the subjects kept a complete record of the food consumed for the specified days and collected specimens as outlined in the experimental procedures.

Basic information on serum lipids and urinary estrogen excretion has been obtained on young women living under normal home conditions who were consuming self-selected diets. The findings in this study showed that the levels of circulating lipids, and fatty acid compositions of serum lipid components appeared to be at least partially affected by endogenous estrogenic hormones. And the excretion of urinary estrogens appeared to be influenced by ovulation and growth and regression of corpus luteum of the menstrual cycle. Decrease of serum lipid levels with the increase of estrogenic hormones production was true during the entire menstrual cycle of nonpregnant young women. This fact may indicate that for women during their reproductive years their own estrogen production can more or less protect them from atherosclerotic changes in the coronary arteries.

As blood samples and urine specimens were only kept at certain day intervals and also only one menstrual cycle for each subject was observed, the true "ovulatory peak" and "luteal maximum" may have been missed for some subjects. Because of the lack of specimens on certain specific dates and only a few analyses for evaluation, one high or one low value could easily skew the data.

SUMMARY

Twelve university women students served as experimental subjects in a study of the serum lipids and urinary excretion of estrogens of healthy nonpregnant menstruating young women who were living under their usual home conditions.

The subjects maintained constant weight on their self-chosen diets during the entire study period. Antecubital blood and 24-hour urine specimens were collected on certain days which represented different stages of the menstrual cycle. Quantitative analyses were made on serum total cholesterol, lipid phosphorus (phospholipid), triglycerides and total lipids. Gas-liquid chromatographic analysis of fatty acid composition of each serum lipid component was also made. Urinary estrone, estradiol and estriol were determined.

Basic data on serum lipid levels, composition of fatty acids of cholesterol esters, phospholipids and triglycerides, and urinary estrogens were obtained on these healthy young women. Findings included the following:

1. The urinary estrogen values showed that estradiol was usually present in the least and estriol in the greatest amounts. The mean values of estrone, estradiol and estriol and total estrogen for the entire study were as follows: 8.7, 4.8, 16.4, and 29.9 μg per 24 hour urine.
2. The menstrual cycle did affect the excretion of estrogen which showed the lowest values during the first week, and then rose to a

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APPENDIX

Appendix A

Tables

Table 21. Serum lipid values on days of menstrual cycle (29 days) of subject AM

Day of Cycle		Total			
Actual	Reference	lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
3	4	518	113.7	147.1	131.5
6	-	522	132.5	224.5	69.0
11	-	459	122.3	159.4	98.1
13	11	292	112.0	117.5	98.4
14	-	474	118.0	131.9	86.9
15	14	425	135.1	128.9	82.8
17	17	420	98.3	145.9	112.6
21	20	556	113.7	142.9	70.2
25	24	527	110.3	153.5	141.0
29	28	474	109.4	163.6	78.6
Mean		467 ± 24[a]	116.5 ± 3.4	151.5 ± 9.3	96.9 ± 7.8
Range		292-556	98.3 - 135.1	117.5 - 224.5	69.0 - 141.0

[a]Standard error of mean.

Table 22. Serum lipid values on days of menstrual cycle (33 days) of subject CO

Day of cycle		Total			
Actual	Reference	lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
2	1	474	133.4	169.9	66.7
12	-	476	152.9	137.4	46.5
13	11	527	139.6	179.5	40.5
14	-	406	117.4	163.2	114.3
15	-	403	114.7	164.4	37.2
16	-	386	175.1	135.6	82.8
22	-	391	123.6	175.9	39.6
24	20	469	146.7	189.7	60.0
29	24	382	136.0	153.6	21.0
30	-	469	143.1	189.1	83.7
Mean		834 $\pm$ 1.6[a]	138.3 $\pm$ 5.7	165.8 $\pm$ 6.1	59.2 $\pm$ 8.9
Range		382-527	114.7 - 175.1	135.6 - 189.7	21.0 - 114.3

[a]Standard error of mean.

Table 23. Serum lipid values on days of menstrual cycle (38 days) of subject CP

Day of cycle		Total			
Actual	Reference	lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
1	1	547	164.3	206.2	110.5
11	-	522	159.8	191.3	82.5
14	-	539	149.7	160.1	139.4
18	-	476	149.3	154.7	110.6
19	8	493	157.0	167.2	81.9
20	-	518	154.7	199.6	63.1
21	-	430	149.7	118.1	85.6
22	11	428	158.9	116.1	145.0
28	17	586	139.2	221.4	109.5
32	20	508	150.7	155.3	86.5
Mean		504 $\pm$ 15[a]	153.3 $\pm$ 2.3	169.0 $\pm$ 11.3	101.5 $\pm$ 8.3
Range		428 - 586	139.2 - 164.3	116.1 - 221.4	63.1 - 145.0

[a]Standard error of mean.

Table 24. Serum lipid values on days of menstrual cycle (29 days) of subject HL

Day of cycle		Total lipid	Cholesterol	Phospholipids	Triglycerides
Actual	Reference				
			mg / 100 ml		
2	1	395	233.1	262.6	113.9
8	-	714	211.1	273.0	82.4
13	-	712	205.8	252.2	102.5
14	11	714	216.3	261.0	98.3
15	-	617	211.1	230.0	133.6
16	14	612	175.4	220.5	88.2
17	-	659	195.3	192.7	187.3
21	20	632	221.6	320.4	115.9
23	24	569	197.4	236.0	115.9
28	28	678	217.4	255.7	106.5
Mean		630 ± 30[a]	208.5 ± 5.1	250.4 ± 10.8	114.5 ± 9.3
Range		395 - 714	175.4 - 233.1	192.7 - 273.0	82.4 - 187.3

[a]Standard error of mean.

Table 25. Serum lipid values on days of menstrual cycle (29 days) of subject JB

Day of cycle		Total			
Actual	Reference	lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
1	1	549	157.4	122.9	116.4
13	14	345	155.6	111.8	104.7
14	-	437	153.7	145.4	139.0
15	-	442	148.2	130.1	301.5
16	17	622	192.6	184.0	145.2
22	-	515	167.6	148.6	232.1
24	24	583	176.9	145.5	172.9
27	28	433	157.4	137.1	-
Mean		491 ± 33[a]	163.7 ± 5.2	140.7 ± 7.6	173.1 ± 26.6
Range		345 - 622	148.2 - 192.6	111.8 - 184.0	104.7 - 301.5

[a]Standard error of mean.

Table 26. Serum lipid values on days of menstrual cycle (31 days) of subject JE

Day of cycle		Total			
Actual	Reference	lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
1	1	423	176.0	106.1	109.5
12	-	517	158.4	110.4	85.8
13	14	330	172.8	95.9	120.6
14	-	496	155.2	123.1	72.3
15	-	569	118.4	140.8	108.0
16	17	488	63.2	108.4	80.7
21	20	472	121.6	107.7	114.6
26	24	500	140.0	104.0	40.8
27	-	423	107.2	107.9	72.3
30	28	413	136.8	101.2	103.8
Mean		463 ± 21[a]	135.0 ± 108	110.6 ± 4.0	90.8 ± 7.9
Range		330 - 569	62.3 - 176.0	95.9 - 140.8	40.8 - 120.6

[a]Standard error of mean.

Table 27. Serum lipid values on days of menstrual cycle (33 days) of subject LR

Day of cycle		Total lipid	Cholesterol	Phospholipids	Triglycerides
Actual	Reference				
			mg / 100 ml		
2	1	484	171.6	179.3	58.7
14	11	646	166.2	835.4	29.6
16	-	556	162.7	181.5	153.3
17	14	722	145.8	257.6	141.1
18	-	610	146.7	230.0	107.4
19	-	542	133.4	196.3	116.9
20	17	649	133.4	204.4	113.3
22	0	595	167.1	207.4	67.6
24	20	714	178.7	201.4	117.9
28	24	488	160.9	129.1	93.6
32	28	639	141.4	204.4	56.9
Mean		604 ± 24[a]	155.3 ± 4.7	202.4 ± 10.1	96.0 ± 11.6
Range		484 - 722	133.4 - 178.7	129.1 - 257.6	29.6 - 153.3

[a]Standard error of mean.

Table 28. Serum lipid values on days of menstrual cycle (23 days) of subject MA

Day of cycle		Total			
Actual	Reference	lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
1	1	445	128.7	179.2	117.8
8	11	527	125.5	179.8	134.1
11	14	537	156.1	208.6	82.1
12	-	440	130.1	203.3	98.3
13	-	496	137.9	211.8	190.1
14	17	513	135.1	201.5	-
15	-	535	136.0	213.0	216.1
16	20	425	141.5	139.9	134.9
22	28	379	111.4	140.3	81.3
Mean		477 ± 19[a]	133.6 ± 4.1	186.4 ± 9.7	131.8 ± 17.4
Range		379 - 537	111.4 - 156.1	139.9 - 213.0	81.3 - 216.1

[a]Standard error of mean.

Table 29. Serum lipid values on days of menstrual cycle (26 days) of subject MC

Day of cycle		Total			
Actual	Reference	Lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
1	1	462	131.8	195.0	55.4
3	4	457	157.1	160.6	53.7
7	8	452	128.3	142.6	59.0
13	14	423	110.9	175.6	76.2
14	-	476	130.1	181.6	65.6
16	17	513	130.1	201.0	45.7
17	-	498	135.3	175.6	49.4
24	24	474	155.4	217.6	58.6
26	28	420	128.3	222.0	62.5
Mean		464 ± 10[a]	134.1 ± 4.8	185.7 ± 8.6	58.5 ± 3.0
Range		420 - 513	110.9 - 157.1	142.6 - 222.0	45.7 - 76.2

[a]Standard error of mean.

Table 30. Serum lipid values on days of menstrual cycle (32 days) of subject MH

Day of cycle		Total lipid	Cholesterol	Phospholipids	Triglycerides
Actual	Reference				
			mg / 100 ml		
1	1	544	141.6	219.6	68.4
12	8	625	172.8	223.3	138.6
18	-	571	213.6	205.9	80.3
20	14	552	206.4	204.8	77.8
25	20	535	217.6	193.0	69.2
26	24	675	223.2	242.4	91.3
32	28	581	164.0	233.2	111.3
Mean		569 ± 11[a]	191.3 ± 11.9	217.4 ± 6.5	91.0 ± 9.7
Range		535 - 625	141.6 - 223.2	193.0 - 242.4	68.4 - 138.6

[a]Standard error of mean.

Table 31. Serum lipid values on days of menstrual cycle (33 days) of subject RM

Day of cycle		Total			
Actual	Reference	lipid	Cholesterol	Phospholipids	Triglycerides
		mg / 100 ml			
1	1	1047	281.6	268.8	147.9
4	4	1050	292.0	330.0	221.4
13	-	967	346.0	291.2	194.4
18	-	785	265.4	202.5	240.9
19	-	948	302.4	216.8	217.2
20	14	979	269.2	218.4	166.8
23	17	982	295.8	235.5	156.0
27	20	819	268.3	212.1	187.5
30	24	875	287.2	266.3	145.8
Mean		939 ± 31[a]	289.8 ± 8.2	249.1 ± 14.3	186.4 ± 11.6
Range		785 - 1050	265.4 - 346.0	202.5 - 330.0	145.8 - 240.9

[a]Standard error of mean.

Table 32. Serum lipid values on days of menstrual cycle (36 days) of subject SL

Day of cycle		Total lipid	Cholesterol	Phospholipids	Triglycerides
Actual	Reference				
			mg / 100 ml		
2	1	490	139.4	139.9	78.4
12	-	447	107.7	110.5	113.9
14	-	496	-	120.5	-
15	-	505	123.1	118.4	93.9
16	8	507	156.5	125.2	87.4
22	-	585	123.1	115.0	162.2
24	14	540	115.4	130.0	91.2
27	17	389	139.4	108.9	65.8
30	20	522	113.7	129.2	200.9
35	28	395	130.8	106.8	159.6
Mean		488 ± 20[a]	127.7 ± 5.1	120.4 ± 3.4	117.0 ± 15.4
Range		389 - 585	107.7 - 156.5	106.8 - 139.9	65.8 - 200.9

[a]Standard error of mean.

Table 33. Urinary estrogen values on day of menstrual cycle (29 days) of subject AM

Day of cycle		Estradiol	Estrone	Estriol	Total
Actual	Reference	(E_2)	(E_1)	(E_3)	(E_t)
			μg / 24-hr.		
3	4	6.0	7.8	12.1	25.9
6	-	4.8	7.7	16.6	29.1
11	-	7.4	9.0	18.2	34.6
13	11	7.5	14.0	27.0	48.5
14	-	10.8	17.0	30.1	57.9
15	14	14.0	20.5	36.3	70.8
16	-	12.8	18.5	42.5	73.8
17	-	7.3	12.6	32.2	52.2
20	20	6.2	11.9	12.1	30.2
21	-	7.0	16.8	14.9	38.7
22	-	10.1	17.2	21.2	48.5
23	-	9.8	18.0	22.3	50.1
24	24	9.5	16.4	22.7	48.6
25	-	6.1	15.5	16.2	37.8
28	-	5.0	10.1	11.9	27.0
29	28	4.3	6.8	10.1	21.2
Mean		8.0 ± .7[a]	13.7 ± 1.1	21.7 ± 2.4	43.4 ± 3.9
Range		4.3 - 14.0	6.8 - 20.5	10.1 - 42.5	21.2-73.8

[a]Standard error of mean.

Table 34. Urinary estrogen values on day of menstrual cycle (33 days) of subject CO

Day of cycle		Estradiol (E_2)	Estrone (E_1)	Estriol (E_3)	Total (E_t)
Actual	Reference				
			μg / 24-hr.		
2	1	N[a]	3.8	10.2	14.0
5	4	0.8	6.2	9.1	16.1
8	-	3.8	4.3	15.3	23.4
12	-	3.2	4.9	19.8	27.9
13	11	3.3	7.4	15.6	26.3
14	-	3.0	6.8	12.5	22.3
15	-	N	5.4	16.8	22.2
16	-	6.8	7.6	20.5	34.9
19	17	1.8	3.5	9.1	14.4
22	20	2.5	4.5	8.1	15.1
23	-	4.2	4.3	12.4	20.9
24	-	3.4	5.4	11.8	20.6
25	-	3.9	6.2	13.7	23.8
28	24	6.2	10.1	24.0	40.3
31	28	2.2	1.9	9.3	13.4
Mean		3.0 ± .5[b]	5.5 ± .5	13.9 ± 1.2	22.4 ± 2.0
Range		N - 6.8	1.9 - 7.6	8.1 - 24.0	13.4 - 40.3

[a] Not detectable.
[b] Standard error of mean.

Table 35. Urinary estrogen values on day of menstrual cycle (38 days) of subject CP

Day of cycle Actual	Day of cycle Reference	Estradiol (E_2)	Estrone (E_1)	Estriol (E_3)	Total (E_t)
			μg / 24-hr.		
1	1	3.2	4.5	7.9	15.6
4	4	4.5	6.4	12.5	23.4
7	-	N[a]	6.6	11.5	18.1
9	-	3.7	7.3	9.6	20.6
11	-	6.0	8.8	18.2	33.0
14	-	5.2	10.9	15.9	32.0
15	-	8.9	10.2	18.5	37.6
18	-	7.3	8.6	18.2	34.1
19	-	4.9	11.4	14.5	30.8
20	8	6.7	10.1	22.8	39.6
21	-	7.4	9.4	23.8	40.5
22	-	8.9	13.4	20.6	42.9
25	-	12.7	13.7	26.8	53.2
27	-	5.3	12.5	22.5	40.3
28	-	6.7	7.1	11.2	25.0
29	17	5.9	7.6	16.9	30.4
32	20	10.1	10.6	31.1	51.8
38	28	2.5	6.8	11.2	20.5
Mean		6.1 ± .7[b]	9.2 ± .6	17.4 ± 1.5	32.7±2.6
Range		N - 12.7	4.5 - 13.7	7.9 - 31.1	15.6 - 53.2

[a]Not detectable. [b]Standard error of mean.

Table 36. Urinary estrogen values on day of menstrual cycle (29 days) of subject HL

Day of cycle		Estradiol	Estrone	Estriol	Total
Actual	Reference	(E_2)	(E_1)	(E_3)	(E_t)
			μg / 24-hr.		
2	1	1.8	4.0	6.5	12.3
5	4	2.1	5.1	6.2	13.4
8	-	3.6	7.0	8.0	18.6
10	8	4.5	8.0	10.8	23.3
11	-	4.9	10.5	12.5	27.9
15	-	6.5	14.8	18.0	39.3
16	14	8.8	20.1	30.8	59.7
17	-	5.2	15.2	28.1	48.5
20	-	2.5	8.7	14.8	26.0
21	-	2.1	8.6	14.2	24.9
22	20	3.0	8.9	15.7	27.6
26	-	4.3	10.8	17.0	32.1
28	28	2.5	7.3	14.0	23.8
Mean		4.0 ± .6[a]	9.9 ± 1.2	15.1 ± 2.0	29.0 ± 3.7
Range		1.8 - 8.8	4.0 - 20.1	6.2 - 30.8	21.3 - 59.7

[a]Standard error of mean.

Table 37. Urinary estrogen values on day of menstrual cycle (29 days) of subject JB

Day of cycle		Estradiol (E_2)	Estrone (E_1)	Estriol (E_3)	Total (E_t)
Actual	Reference				
			μg / 24-hr.		
1	1	N[a]	1.8	2.5	4.3
7	8	N	2.8	2.0	4.8
10	11	.7	5.0	2.2	7.9
13	14	6.0	10.6	16.8	33.4
14	-	2.0	5.0	7.5	14.5
15	-	.9	4.1	7.2	12.2
16	17	.2	5.2	8.0	13.4
17	-	.5	5.7	6.9	13.1
21	-	N	4.1	11.2	15.3
22	-	1.1	6.7	12.5	20.3
23	24	1.8	8.5	13.1	23.4
24	-	.8	7.1	11.9	19.8
25	-	.6	5.7	10.3	16.6
27	28	N	3.1	4.5	7.6
Mean		1.0 ± .4[b]	5.4 ± .6	8.3 ± 1.2	14.8 ± 2.1
Range		N - 6.0	1.8 - 10.6	2.0 - 16.8	4.3 - 33.4

[a]Not detectable.
[b]Standard error of mean.

Table 38. Urinary estrogen values on day of menstrual cycle (31 days) of subject JE

Day of cycle		Estradiol (E_2)	Estrone (E_1)	Estriol (E_3)	Total (E_t)
Actual	Reference				
			μg / 24-hr.		
2	1	3.2	4.1	12.0	19.3
4	4	4.0	4.8	10.9	19.7
7	-	4.8	8.1	15.1	28.0
10	8	7.5	12.0	16.7	36.2
12	-	7.8	11.8	14.8	34.4
13	11	8.5	13.1	19.3	40.9
14	-	7.1	15.8	24.1	47.0
15	-	10.0	17.2	27.5	54.7
16	14	11.3	21.0	30.7	63.0
18	-	9.0	15.3	28.2	52.5
20	17	7.3	11.0	17.3	35.6
25	-	6.3	17.8	26.5	50.6
26	24	7.9	18.1	24.8	50.8
30	28	4.8	6.7	13.4	24.9
Mean		7.1 ± .6[a]	12.6 ± 1.4	20.1 ± 1.8	39.8 ± 3.7
Range		3.2 - 11.3	4.1 - 21.0	10.9-30.7	19.3-63.0

[a]Standard error of mean.

Table 39. Urinary estrogen values on day of menstrual cycle (33 days) of subject LR

Day of cycle		Estradiol (E_2)	Estrone (E_1)	Estriol (E_3)	Total (E_t)
Actual	Reference				
			μg / 24-hr.		
2	1	N[a]	4.1	6.8	10.9
4	4	4.1	6.2	3.2	13.5
7	-	5.5	7.4	11.0	23.9
13	11	6.7	8.9	17.0	32.6
15	-	2.3	8.3	21.3	31.9
16	-	N	9.0	23.9	32.9
17	14	7.3	16.4	34.9	58.6
18	-	5.9	11.1	22.8	39.8
19	-	5.0	13.7	11.5	30.2
20	17	3.6	9.4	15.2	28.2
21	-	3.1	9.3	15.9	28.3
22	-	2.3	7.6	12.4	22.3
26	24	6.6	10.9	31.1	48.6
30	28	1.8	7.2	7.9	16.9
Mean		3.8 ± .6[b]	9.3 ± .8	16.8 ± 2.4	29.9 ± 3.5
Range		N - 7.3	4.1 - 16.4	3.2 - 34.9	10.9 - 58.6

[a]Not detectable.
[b]Standard error of mean.

Table 40. Urinary estrogen values on day of menstrual cycle (23 days) of subject MA

Day of cycle		Estradiol (E_2)	Estrone (E_1)	Estriol (E_3)	Total (E_t)
Actual	Reference				
			μg / 24-hr.		
1	1	2.1	4.3	6.0	12.4
4	4	N[a]	3.8	4.1	7.9
7	-	4.8	6.5	8.3	19.6
8	11	4.8	7.1	9.8	21.7
9	-	4.6	7.3	8.7	20.6
11	14	6.2	11.8	34.6	52.6
12	-	5.1	10.1	36.3	51.5
13	-	1.8	6.8	25.1	33.7
14	17	2.3	6.1	19.8	28.2
15	-	2.9	6.6	15.0	24.5
16	20	2.1	5.9	14.7	22.7
22	-	3.1	5.3	10.3	18.7
23	28	3.7	6.2	8.5	18.4
Mean		3.3 ± .5[b]	6.7 ± .6	15.5 ± 2.9	25.6 ± 3.9
Range		N - 6.2	3.8 - 11.8	4.1 - 36.3	7.9 - 52.6

[a] Not detectable.
[b] Standard error of mean.

Table 41. Urinary estrogen values on day of menstrual cycle (26 days) of subject MC

Day of cycle		Estradiol	Estrone	Estriol	Total
Actual	Reference	(E_2)	(E_1)	(E_3)	(E_t)
			μg / 24-hr.		
3	-	2.3	4.2	5.0	11.5
4	4	1.8	4.5	5.3	11.6
7	8	2.5	6.2	8.1	16.8
10	11	3.8	8.1	10.7	22.6
13	14	8.0	20.2	29.5	57.7
14	-	5.7	14.5	28.1	48.3
15	-	2.6	10.1	20.2	32.9
16	17	3.3	9.5	17.0	29.8
17	-	3.1	9.7	14.1	26.9
20	-	3.7	10.9	15.0	29.6
23	24	2.8	10.2	17.8	30.8
24	-	2.9	10.3	18.1	31.3
25	-	2.7	9.2	13.3	25.2
26	28	2.4	7.2	12.8	22.4
Mean		$3.4 \pm .4$[a]	9.6 ± 1.1	15.4 ± 1.9	28.4 ± 3.4
Range		1.8 - 8.0	4.2 - 20.2	5.0 - 29.5	11.5-57.7

[a]Standard error of mean.

Table 42. Urinary estrogen values on day of menstrual cycle (32 days) of subject MH

Day of cycle		Estradiol	Estrone	Estriol	Total
Actual	Reference	(E_2)	(E_1)	(E_3)	(E_t)
			μg / 24-hr.		
2	1	3.3	4.5	7.9	15.7
9	-	3.7	6.6	10.3	20.6
12	8	5.9	10.6	11.2	27.7
14	-	7.3	13.7	16.9	37.9
17	-	6.2	9.4	19.8	35.4
18	-	8.9	10.2	22.5	41.6
19	14	5.2	13.4	31.1	49.7
20	-	6.0	13.3	18.5	37.8
23	-	1.4	7.7	11.5	20.6
25	20	5.2	10.2	18.5	33.9
26	24	4.9	9.4	29.4	43.7
27	-	3.7	7.9	26.8	38.4
28	-	3.4	7.3	13.5	24.2
32	28	2.5	5.2	9.2	16.9
Mean		4.8 ± .5[a]	9.2 ± .8	17.7 ± 2.0	32.8±2.8
Range		1.4 - 8.9	4.5 - 13.4	7.9 - 31.1	15.7-49.7

[a]Standard error of mean.

Table 43. Urinary estrogen values on day of menstrual cycle (33 days) of subject RM

Day of cycle		Estradiol	Estrone	Estriol	Total
Actual	Reference	(E_2)	(E_1)	(E_3)	(E_t)
			μg / 24-hr.		
11	8	2.5	7.1	15.3	24.9
13	-	5.1	6.8	15.9	27.8
16	11	3.7	5.8	12.0	21.5
18	-	1.7	9.5	16.0	27.2
19	-	6.2	8.2	16.8	31.2
23	17	3.4	6.8	9.1	19.3
26	24	5.3	8.8	14.7	28.8
27	-	3.8	9.2	13.6	26.6
Mean		4.0 ± .5[a]	7.8 ± .5	14.2 ± .9	25.9 ± 1.4
Range		1.7 - 5.3	5.8 - 9.5	9.1 - 15.9	19.3 - 31.2

[a]Standard error of mean.

Table 44. Urinary estrogen values on day of menstrual cycle (36 days) of subject SL

Day of cycle		Estradiol	Estrone	Estriol	Total
Actual	Reference	(E_2)	(E_1)	(E_3)	(E_t)
			μg / 24-hr.		
1	1	N[a]	4.1	6.8	10.9
4	4	5.0	6.2	14.5	25.7
7	-	7.2	9.0	11.5	27.7
10	-	5.9	7.7	15.9	29.5
12	-	5.5	7.4	16.9	29.8
13	-	7.3	7.6	15.2	30.1
14	-	6.7	9.3	20.6	36.6
15	-	4.1	8.3	21.3	33.7
16	-	3.0	11.1	18.6	32.7
18	8	3.6	7.6	27.8	39.0
21	11	6.7	10.9	22.8	40.4
22	-	5.2	10.2	35.2	50.6
23	-	7.9	8.3	34.9	51.1
24	14	9.4	16.4	40.4	66.2
27	17	2.3	3.6	11.2	17.1
Mean		5.3 ± .6[b]	8.5 ± .8	20.9 ± 2.5	34.7 ± 3.6
Range		N - 9.4	3.6 - 16.4	6.8 - 40.4	10.9 - 66.2

[a]Not detectable.
[b]Standard error of mean.

Appendix B

Dietary Record Form

Name_____ Date_____ Day of week____

	Breakfast	Lunch	Dinner	Snacks
1. Milk				
2. Vegetables, raw				
Cooked				
4. Fruit				
5. Bread				
6. Crackers, rice, cereal, pastes				
7. Meat, in ounces or slices				
8. Egg				
9. Fat, butter or oil				
10. Mixed dishes[a]				
11. Desserts				
12. Sugar				
13. Candy				
14. Soft drinks				
15. Supplements				

[a] Put recipes or ingredients on back of sheet.

Appendix C

Analytical Methods

1. Extraction of total lipids from serum

The method of Folch, Lees and Sloane-Stanley (1957) modified by Smith (1965) with slight modification.

Reagents

1. Chloroform-methanol, 2:1 (v/v). Mix 2 volumes chloroform with 1 volume methanol.
2. Petroleum ether.
3. Anhydrous sodium sulfate.

Equipment

1. Water bath.
2. Magnetic stirrer.

Procedure

1. The frozen serum samples are incubated at 37 C in a water bath for 30 min, cooled to room temperature and mixed gently with a buzzer.
2. One volume of serum is added dropwise to 20 volumes of chloroform-methanol in a beaker while the solvent is kept in motion by use of a magnetic stirrer.
3. The lipid extract is filtered through fat-free glass wool into an Erlenmeyer flask.

4. The precipitate is washed into the beaker with 20 volumes of fresh solvent, boiled, cooled, and filtered into the original filtrate.

5. 20% by volume of redistilled water is added to the lipid extract and mixed thoroughly. The flask is stoppered and allowed to stand until two distinct layers clearly separate.

6. The water layer on the top is removed by aspiration and discarded, and the solvent is dried by adding anhydrous sodium sulfate to the lipid extract.

7. The flask is flushed with nitrogen and stoppered with a glass stopper and is then held overnight at -5 C.

8. Next day, the lipid extract is filtered through fat-free glass wool into a 25 x 175 mm tube to remove the sodium sulfate.

9. The solvent is evaporated to dryness under a stream of nitrogen gas.

10. The lipid is immediately dissolved in a small volume of petroleum ether and is quantitatively transferred to a one-milliliter volumetric flask.

2. Determination of total lipids

The method of Bragdon (1951) with slight modification.

Reagents

1. Color reagent. 20 g $K_2Cr_2O_7$ crystals, c.p. is powdered in a mortar and added slowly with shaking to 1000 ml of sulfuric acid, c.p. (sp. gr. 1.84) maintained at a temperature not exceeding 100 C. There should be no undissolved residue. If the reagent is protected from contamination and from exposure to direct sunlight, it darkens only very slowly with age.

2. Standard solution. Dissolve appropriate amount of tripalmitin in solvent to prepare different concentrations of standard solutions.

Equipment

1. Beckman DU spectrophotometer.

2. Water bath.

Procedure

1. 100 μl of lipid extracts in a 10 ml volumetric flasks are evaporated under nitrogen at a temperature under 60 C.

2. 4 ml color reagent is added. Then the flasks are stoppered and sealed with a drop of reagent, and placed in boiling water for 30 min.

3. An appropriate blank is similarly prepared with 4 ml color reagent.

4. The flasks are cooled in water and about 5 ml redistilled water is added.

5. The flasks are restoppered, recooled, and brought to final volume in a water bath at 25 C.

6. After 30 min absorbance is read against blank.

7. A standard curve is prepared by using different concentrations of tripalmitin, and the slope of the curve is determined.

Calculation

Mg of total lipid per 100 ml of serum

$$= \text{Density of unknown} \times \text{Factor} \times \frac{100}{\text{volume of unknown}}$$

Factor: slope of standard curve.

3. Determination of cholesterol

The method of Clark, Rubin and Arthur (1968).

Reagents

1. Absolute ethanol, reagent grade.

2. KOH-ethanol reagent, 2 ml 33% aqueous KOH in 100 ml absolute ethanol.

3. Methylene chloride, Baker Analyzed reagent.

4. Concentrated sulfuric acid, Baker and Adams, C. P. reagent.

5. Iron-ethanol reagent (FeAlc), 5.0 gm reagent-grade $FeCl_3 . 6H_2O$ is dissolved in 1000 ml absolute ethanol (may be stored indefinitely at room temperature).

6. Cholesterol working standard, 100 mg cholesterol dissolved in 90 ml absolute ethanol in a 100 ml volumetric flask, made to volume with additional ethanol; 2 ml aliquots are transferred to vials, capped, and stored at -20 C. A fresh vial of standard (1 mg/ml) is used each week.

Equipment

1. Beckman model DU spectrophotometer.

2. Beckman Quartz cells with 10 mm light path.

3. International model PR-2 centrifuge.

4. Water bath.

Procedure

1. With a Hamilton syringe, 50 µl of well-mixed serum or plasma is transferred to the bottom of a 20 ml tube. The syringe is rinsed with water between samples. A standard and a blank are likewise prepared with 50 µl of cholesterol standard solution and 50 µl of water, respectively.

2. FeAlc reagent (2 ml) is added to each tube with a volumetric pipet; the tubes are swirled gently during addition. Then 1.0 ml KOH-ethanol reagent is added to each tube with a volumetric pipet. The tubes are capped, vigorously shaken, and placed in a 65 C water bath for 5 min to aid protein-$Fe(OH)_3$ precipitation.

3. The tubes are centrifuged for 5 min at 2,000 rpm to separate the precipitate.

4. 1.0 ml of each supernatant is transferred to each of two clean 20 ml tubes with a volumetric pipet.

5. 1.0 ml FeAlc reagent is pipetted into each tube and gently shaken.

6. 2.0 ml concentrated sulfuric acid is gently layered beneath the alcoholic solution by pipetting the acid slowly down the side of the tube. Any tube in which accidental mixing occurs is discarded.

7. The tubes are carefully capped and vigorously shaken several times by hand, or mixed well by vortex mixer. The hot solutions are allowed to cool to room temperature (20 min). A red-orange color develops.

8. An additional 2 ml concentrated sulfuric acid is gently layered beneath the colored solution. Without mixing, 5.0 ml methylene chloride is added with a volumetric pipet.

9. The tubes are capped and shaken vigorously by hand for 2 min or for 5 min on an automatic shaker. The solutions are allowed to stand for 10 min to separate the phases. A portion of the methylene chloride phase (upper layer) of the reagent blank is transferred to a spectrophotometer cell. The spectrophotometer is set to 100% transmission at 525 mμ with the reagent blank against distilled water in the reference cell. The optical densities of the methylene chloride phases of the sample and standard tubes are then read against the distilled water reference. Each tube is left capped until it is read to prevent absorption of atmospheric water and evaporation of methylene chloride.

Calculation

Mg cholesterol per 100 ml serum

$$= \frac{\text{Optical density of unknown}}{\text{Optical density of standard}} \times \frac{100}{\text{volume of unknown}}$$

x mg cholesterol in standard.

4. Determination of lipid phosphorus

The method of Baginske, Foa, Weiner and Zak (1968).

Reagents

1. Trichloroacetic acid (TCA), 10% (w/v).

2. Ascorbic acid-trichloroacetic acid reagent (A-TCA). Dissolve 1.0 g of ascorbic acid in 100 ml of 10% trichloroacetic acid.

3. Arsenite-citrate solution (AC). Dissolve 2.0 g of sodium citrate dihydrate and 2.0 g of sodium arsenite in 100 ml of 2% acetic acid.

4. Ammonium molybdate solution (AM). Dissolve 1.0 g of ammonium molybdate tetrahydrate in 100 ml of distilled water.

5. Nitric acid-calcium nitrate solution(NAC). Dissolve 50 mg of $CaCO_3$ in 1 liter of HNO_3.

6. Phosphate stock standard (1 mg of P/ml). Dissolve 4.394 g of KH_2PO_4 in distilled water, using a 1-liter volumetric flask.

7. Phosphorus working standard (5.0 mg/100 ml distilled H_2O).

8. Alcohol-ether, 3:1 (A-E). Mix 3 volumes absolute ethanol with 1 volume ethyl ether.

Equipment

1. Micro-Kjeldahl digestion unit.

2. Centrifuge.

3. Beckman DU spectrophotometer.

4. Water bath.

Procedure

1. Pipet 0.1 ml of the sample slowly into 2.0 ml AE in a test tube, stopper the tube, mix, and centrifuge at 3000 rpm for 2 min.

2. Evaporate 1.0 ml of the supernatant fluid (or total lipid extract 50 μl) to dryness in a 25 x 150 mm borosilicate glass test tube with the aid of a warm water bath.

3. Place 2 boiling granules into the tube after all solvent has evaporated. Add 2.0 ml of NAC, 2 boiling stones, and heat in the Kjeldahl until all yellow fumes have disappeared to ensure complete elimination of all oxides of nitrogen.

4. Prepare the standard by pipetting 0.1 ml working standard into 2 ml NAC. The reagent blank contains only NAC. Process both tubes in the same manner as the sample.

5. Dissolve the dried residues with 1.0 ml A-TCA. Add 0.5 ml AM, mix; add 1.0 ml AC and mix.

6. After 15 min., determine the absorbance against the reagent blank at either 700 mμ or 840 mμ.

Calculation

Mg of lipid phosphorus per 100 ml of serum

$$= \frac{\text{Optical density of unknown}}{\text{Optical density of standard}} \times \frac{100}{\text{Volume of unknown}}$$

x mg phosphorus in standard

Mg of phospholipid per 100 ml serum = mg lipid phosphorus x 25.

5. Determination of triglycerides

The method of Galletti (1967).

Reagents

All reagents and solvents are analytical reagent grade.

1. Isopropyl ether (peroxide free), purified by passage through a column of activated alumina. Store in ice box.

2. Silicic acid (100 mesh, suitable for chromatography) activated overnight at 170 C.

3. Ethanolic potassium hydroxide, 0.05 M: dilute 1 ml of aqueous 5 M potassium hydroxide to 100 ml with 95% v/v ethanol.

4. Phosphoric acid, 4 N.

5. Potassium metaperiodate, 0.015 M: dissolve 345 mg of potassium metaperiodate in 100 ml of distilled water.

6. Phenylhydrazine hydrochloride, 1% w/v in water. Prepared immediately before use.

7. Potassium ferricyanide, 1% w/v in water.

8. Sulfuric acid and glacial acetic acid.

9. Isopropyl alcohol.

10. Standard solution of tripalmitin: approximately 30 mg of pure tripalmitin is weighed accurately and dissolved in 100 ml of isopropyl ether.

11. Working standard solution: Dilute standards are prepared containing 3 to 30 μg/0.5 ml, corresponding to the range 60-600 mg glyceride per 100 ml serum.

Equipment

1. Beckman DU spectrophotometer.

2. Centrifuge.

3. Shaker.

4. Water bath.

5. Vortex mixer.

Procedure

1. A spoonful of silicic acid (approximately 500 mg) is placed in a glass-stoppered tube and wetted with 0.5 ml isopropyl ether.

2. 40 μl serum is added on the top of the silicic acid and followed by an additional 3.5 ml isopropyl ether.

3. The tube is vigorously shaken horizontally for half an hour.

4. Centrifuged 5 min at 2000 rpm.

5. A blank (0.5 ml of isopropyl ether) and working standard solutions are evaporated to dryness and run simultaneously through the whole procedure.

6. 0.25 ml of ethanolic KOH solution is added to each tube, then the tubes are firmly stoppered and placed in a 60 C water bath for 20 min.

7. After cooling, 0.05 ml of 4 N phosphoric acid and 0.1 ml potassium metaperiodate solution are added.

8. After 15 min 0.2 ml phenylhydrazine hydrochloride solution is added.

9. After 10 min 0.2 ml potassium ferricyanide solution is added.

10. Immediately the tubes are placed into an ice bath for exactly 5 min.

11. The tubes are removed from the bath, 1 ml of sulfuric-acetic reagent and 1 ml isopropyl alcohol are added, mixing after each addition. Work with one at a time.

12. 20 min later the absorbance is read at 520 mμ against the blank.

Calculation

Mg of triglycerides per 100 ml of serum

$$= \frac{\text{Optical density of unknown}}{\text{Optical density of standard}} \times \frac{100}{\text{volume of unknown}}$$

x mg tripalmitin in standard.

6. Quantitation of lipid classes

The methods of Maier and Mangold (1964) and Pie and Giner (1966).

Reagents

1. Silica gel H[1]
2. Iodine
3. Acetone, reagent grade; redistilled
4. Glacial acetic acid
5. Skelly Solve B (Petroleum ether), distilled and the fraction distilling between 66-69 C is collected.
6. Diethyl ether.
7. Developing solvent.
8. Methanol, absolute. Methanol is distilled over potassium hydroxide.
9. Chloroform, reagent grade.
10. Nitrogen gas.
11. Lipid standards for thin-layer chromatography.[2]

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[1] Brinkman Instruments Inc., Cantiague Rd., Westbury, New York 11590.

[2] The Hormel Institute, University of Minnesota, 801, 16th Ave., N. E., Austin, Minnesota.

Equipment

1. Desaga[1] equipment; basic outfit No. 601. The following items are included in the basic outfit:

Spreader No. 611 (Brinkman Model II) with adjustable slit width.

1 plastic aligning tray for coating 5 plates 20 x 20 cm.

10 glass plates of uniform thickness (4 mm) 20 x 20 cm, and two end plates 5 x 20 cm.

1 rectangular chromatography tank and lid to take 20 x 20 cm plates.

1 spotting template of transparent plastic, with scale.

1 light metal drying rack to take 10 plates 20 x 20 cm.

2 graduated micropipettes of 10 µl capacity for applying the mixture of substances.

Procedure

1. Setting out the Glass Plates and Preparing the Spreader

The plates are carefully cleaned and completely free from grease.

The aligning tray, approximately 110 cm long, is laid on the bench with its short rim on the right. Five 20 x 20 cm plates (correspondingly more if narrower plates are used) of equal thickness are placed on the tray. To prevent them from sliding about on the baseboard they can be stuck down with a little water.

The left- and right-hand ends of the row of plates are completed with two 50 x 20 cm plates (end plates). The top of the spreader is opened and it

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[1]Desaga GmbH., Heidelberg, Germany. Agents in the USA: C.A. Brinkman Co., Inc., Great Neck, Long Island, N. Y.

is then placed on the left-hand end-plate with lever of the tipping mechanism pointing to the right, as indicated by the red arrow engraved on the upper side of the apparatus. It lies with its guide bar pressed close to the tray.

2. Preparing the suspension and filling the spreader

27 g of Silica Gel H is mixed with 72 ml redistilled water by grinding in a mortar or by vigorous shaking (for 30 to 45 sec) in a stoppered conical flask (200 to 250 ml). Owing to its plaster of Paris content, the fairly thin suspension sets within a few minutes. It is, therefore, immediately transferred to the open spreader, on which the desired layer thickness has previously been set.

3. Coating the plates

When the suspension has been poured into the spreader, the lever is turned to the left through 180^{o} so that the opening necessary to admit the air is visible at red arrow. When the slurry can be seen coming out, the process of coating is begun. For this it is best to stand by the center of the tray, hold the spreader with both hands and draw it across the plates to the further end-plate without applying much pressure. When the end-plate has been reached the lever is again turned to the right, which prevents any liquid still remaining in the spreader from running out.

When the plates have been coated, the cover screw by the side of the arrow is undone, and the tipping mechanism is taken out for cleaning. The parts of the apparatus are thoroughly brushed under running water and then rinsed with distilled water.

4. Treatment of the plates after coating

Drying: The plates are left in position until their surface has become completely mat (About 10 min). Leave them to dry in air overnight.

Activation: The plates are placed in a drying cabinet. Heating to 110 C for 2 hours.

Storage: The active plates are stored over a desiccant in a desiccator, or in a plate cabinet. The layers must naturally be protected as well as possible against laboratory fumes and mechanical damage.

5. Applying sample and separating lipid classes

a. The total lipid extract, dissolved in petroleum ether, is applied to coated plate with micro pipet at a point approximately two cm from the edge of the plate at right angles to the direction in which the plates are coated.

b. A lipid standard prepared for TLC is applied to coated plate layer as a marker for identifying the lipid classes.

c. Immediately after the sample is applied, the plate is placed in a developing tank which previously has been saturated by adding 100 ml of developing solvent. Saturation is accomplished by lining the tank with a filter paper wick.

d. When the solvent front has reached a pre-determined mark, the plate is removed from the tank.

e. The plate is air dried, and quickly put in an iodine atmosphere until the lipid components become visible (yellow spots).

f. The plate is removed from the iodine atmosphere.

g. The areas of particular lipid classes are located.

h. The portion of Silica Gel H containing the respective lipid component is immediately scraped off into a flask containing eluting solvent. Diethyl ether is used for elution of cholesterol esters and triglycerides. Methanol is used for the elution of phospholipids.

i. The Silica Gel H lipid mixture is filtered through a sintered glass filter into a tube. The flask is rinsed several times with solvent to insure quantitative transfer of the lipid.

j. The solvent is evaporated just to dryness in a nitrogen atmosphere while the tube is held in a warm water bath.

k. The lipid is dissolved in petroleum ether and quantitatively transferred to a tube which is stoppered with a cork stopper.

l. The sample is held at -10 C until the time of preparation for gas-liquid chromatographic analysis.

7. Gas-liquid chromatographic analysis of methyl esters of fatty acids

The method of Stoffel, Chu and Ahrens (1959) and modified by Smith (1965).

Reagents

1. Methanol, absolute, distilled over potassium hydroxide.

2. Hydrogen chloride in superdry methanol, 5% solution. A weighed amount of hydrogen chloride gas is bubbled into a weighed amount of methanol.

3. Benzene, distilled.

4. Skelly Solve B (petroleum ether) distilled and the fraction distilling between 66-69 C is collected.

5. Chloroform, distilled. Five-tenths to 1 percent ethanol is added as a preservative.

6. Nitrogen gas.

7. Sodium sulfate, anhydrous.

8. Lipid standards for gas-liquid chromatography.[1]

Equipment

1. Gas-liquid chromatographic equipment: Aerograph Hi-Fi Model 600 C, equipped with a flame ionization detector.

2. Stainless steel column, one-eighth inch inner diameter, 7.5 feet long and packed with 20% DEGS 60/80 mesh acid washed chromsorb W.

3. Heating block.

4. Microsublimation tube.

5. Water bath.

6. Centrifuge.

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[1]The Hormel Institute, University of Minnesota, 801, 16th Ave., N. E., Austin, Minnesota.

Procedure

1. 1 to 3 mg of sample is dissolved in a mixture of 4 ml 5% HCl-methanol and 2 ml of benzene in a microsublimation tube to which a condenser is connected. Boiling chips are used to prevent bumping.

2. The tubes are placed in a heating block and refluxed at 90-100 C in a nitrogen atmosphere for 2 hours for the interesterification of triglycerides and 6 hours for the cholesterol esters and phospholipids.

3. The samples are shaken frequently at the beginning of the refluxing period to dissolve the lipid mixture.

4. At the end of the refluxing, the mixture is evaporated under a stream of nitrogen while the tubes are held in a warm water bath.

5. A small amount of benzene is added to the tubes to remove any water in the sample. Drying the sample with benzene is repeated if necessary.

6. The esters are dissolved in a small volume of petroleum ether (phospholipids are dissolved in a small volume of methanol) and quantitatively transferred to a tube.

7. The samples are further dried with anhydrous sodium sulfate and then centrifuged for ease in concentrating the water and sodium sulfate in the bottom of the tube.

8. The solvent is evaporated in preparation for injection of the sample.

9. The methyl ester sample is dissolved in 10 μl of chloroform and injected into the column using a microliter syringe. (The column is coiled in 2 1/4 cm diameter coils and conditioned for 24 hours before samples are injected. The temperature of the column is 190 to 195 C).

10. The rate of flow of the nitrogen carrier gas is adjusted to give good resolution of the methyl esters of the fatty acids.

11. Standard mixtures of methyl esters of fatty acids are analyzed at frequent intervals for identification of the various fatty esters and to determine the degree of quantitative resolution obtained with the specific column and instrument in use.

Calculations

The proportional composition of the fatty acids in the total sample is obtained by the multiplication of peak height by width at half-height of the area associated with each component and calculation of the percentage of the total area. The amounts of fatty acids calculated as components of several standard mixtures varied no more than 1 percent of the actual values.